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Development of a novel biomaterial: Part III. Stability and hydration in various media

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Defence R&D Canada – Toronto
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Abstract

This report describes the stability and hydration properties of gelatin and gelatin-HydroThane™ composite biomaterials in different media. A Gel permeation chromatography (GPC) method and swelling measurements were applied to examine the stability of the tested biomaterials. The degradation of gelatin in distilled water and 0.1% sodium azide aqueous solution was confirmed by the GPC study. The degradation in terms of changes in both molecular weight and its distribution was increased with increasing temperature. Samples of gelatin-HydroThane™ composite biomaterials were prepared by photochemical crosslinking of gelatin and HydroThane™ in dimethylsulfoxide solution under different conditions. Results of the stability and hydration studies in the aqueous solution demonstrated that the freeze-dried composite biomaterial exhibited relatively stable swelling for more than 30 days, but a loss of the gelatin component occurred, approximately by 10% at room temperature and 50% at 37°C. Similar swelling study in a serum-containing medium indicated that the composite biomaterial would maintain the hydration stability for its potential application in a wound dressing. A number of key factors in the preparation of the biomaterial were investigated to optimize its hydration properties. The composite biomaterial showed good mechanical properties, primarily conferred by the more stable HydroThane™ component. The results of this study provide a better understanding of the physicochemical properties of a gelatin-HydroThane™ composite biomaterial, which holds much promise for its further development into a dressing for wound management and other biomedical applications.

Résumé

Dans le présent rapport, on décrit les propriétés de stabilité et d'hydratation de biomatériaux à base de gélatine et de composite gélatine-HydroThane^{MC}, dans divers milieux. On a utilisé une méthode de chromatographie par perméation sur gel (CPG) et on a fait des mesures de gonflement pour étudier la stabilité de ces biomatériaux. L'étude par CPG a permis de confirmer que la gélatine se dégrade dans l'eau distillée et dans une solution aqueuse à 0,1 % d'azoture de sodium. La dégradation, en termes de modification des poids moléculaires et de modification de la distribution de ses poids, était plus importante lorsqu'on augmentait la température. On a préparé des échantillons de biomatériaux composites gélatine-HydroThane^{MC} par réticulation photochimique de gélatine et d'HydroThane^{MC} dans une solution de diméthylsulfoxyde, dans diverses conditions. Les résultats obtenus lors des études en solution aqueuse sur la stabilité et l'hydratation ont montré que le biomatériau composite lyophilisé exhibait un gonflement relativement stable pendant plus de 30 jours, mais qu'une perte du composant gélatine survenait, d'environ 10 % à température ambiante et d'environ 50 % à 37 °C. Une étude similaire réalisée dans un milieu contenant du sérum a permis de montrer que le biomatériau composite conserverait sa stabilité à l'hydratation pour son application potentielle à un pansement. On a étudié un certain nombre de paramètres clés de la préparation de ce biomatériau, afin d'optimiser ses propriétés d'hydratation. Le biomatériau composite a exhibé de bonnes propriétés mécaniques, principalement dues au composant d'HydroThane^{MC} plus stable. Les résultats de la présente étude permettent d'avoir une meilleure compréhension des propriétés physico-chimiques d'un biomatériau composite gélatine-HydroThane^{MC}, qui semble très prometteur pour un futur développement en tant que pansement pour le traitement de blessures ou pour d'autres applications biomédicales.

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Executive summary

In support of MOM Thrust 6c under WBE 16ci04 (Emerging Material Technologies for Applications in Battlefield Wound Care), DRDC Toronto is developing a novel hydrogel-elastomer interpenetrating polymer network (IPN) biomaterial for use as a wound dressing on the battlefield. This technical report summarizes a study to characterize the *in vitro* stability and hydration of gelatin and gelatin-HydroThane™ composite biomaterials for their potential application as a dressing for treating battlefield wounds. All studies, including method development; material preparations; and testing and evaluations were conducted at DRDC Toronto.

A gel permeation chromatography (GPC) system was established and validated to study the stability of gelatin in aqueous solutions. GPC was also used to quantify and understand gelatin loss from its composite biomaterials in aqueous solutions, including a bovine serum-containing medium. Acetonitrile was used to remove the adsorbed serum proteins from the incubated material, but the residual serum proteins interfered with subsequent gelatin characterizations. Additional separation methods, other than the GPC system, need to be developed to monitor the stability of the composite material in the serum medium.

We successfully developed a biomaterial using a proprietary hydrogel-elastomer interpenetrating polymer network (IPN), which can be potentially applied to form a dressing for wound management. A large number of the composite biomaterials were prepared under different conditions. In the course of this study, we also developed two novel methods, through freezing and aging certain components (e.g., methacrylated gelatin solution) of the composite material, to provide an improvement to the finished biomaterial product.

Swelling measurement was extensively used as a simple method to characterize the biomaterial's hydration values and indicate the swelling stability. In addition, mechanical testing was used to examine the stability of the HydroThane™ component in the composite biomaterial. Our results, based on the hydration and mechanical stability studies, show that the biomaterial is suitable for its potential application in wound dressings.

In summary, the *in vitro* stability and hydration of gelatin and the composite biomaterial have been well characterized. This novel DRDC composite biomaterial can be further developed and exploited to produce dressings for battlefield wound management.

Peng, H., Martineau, L., Shek, P.N. 2005. Development of a novel biomaterial: Part III. Stability and hydration in various media. DRDC Toronto TR 2005-252. Defence R & D Canada—Toronto.

Sommaire

En soutien au vecteur de projet MOM 6c, dans le cadre de WBE 16ci04 (Emerging Material Technologies for Applications in Battlefield Wound Care), RDDC Toronto développe un nouveau réseau de polymères interpénétrants (RPI) de type hydrogel-élastomère, devant servir de pansement sur le champ de bataille. Le présent rapport technique résume une étude ayant pour objectif la caractérisation de la stabilité et de l'hydratation *in vitro* de biomatériaux à base de gélatine et de composite gélatine- HydroThane^{MC}, à des fins d'application potentielle comme pansement pour le traitement de blessures sur le champ de bataille. Toutes les études, y compris le développement de méthodes, la préparation des matériaux, leurs tests et leurs évaluations, ont été réalisées à RDDC Toronto.

On a mis sur pied et validé un système de chromatographie par perméation sur gel (CPG) pour étudier la stabilité de la gélatine dans des solutions aqueuses. On a aussi utilisé la CPG pour quantifier et comprendre la perte de gélatine par les biomatériaux composites en solutions aqueuses, y compris dans un milieu contenant du sérum bovin. On a utilisé de l'acétonitrile pour éliminer les protéines de sérum adsorbées du matériau incubé. Toutefois, le sérum résiduel a provoqué des interférences lors de caractérisations ultérieures de la gélatine. Il faudra développer d'autres méthodes de séparation, autres que le système de CPG, pour suivre la stabilité du matériau composite dans le milieu à base de sérum.

On a réussi à développer un biomatériau à partir d'un réseau de polymères interpénétrants (RPI) breveté de type hydrogel-élastomère. Ce biomatériau peut potentiellement servir à la confection d'un pansement pour le traitement de blessures. On a préparé un grand nombre des biomatériaux composites dans diverses conditions. Au cours de la présente étude, on a aussi développé deux nouvelles méthodes, par congélation et vieillissement de certains composants (p. ex. solution de gélatine méthacrylée) du matériau composite, afin d'améliorer le produit fini.

On a largement utilisé des mesures de gonflement comme méthode simple pour caractériser les valeurs d'hydratation du biomatériau et déterminer la stabilité au gonflement. De plus, on a fait des tests mécaniques pour étudier la stabilité du composant HydroThane^{MC} dans le biomatériau composite. Nos résultats, basés sur les études de stabilité mécanique et d'hydratation, montrent que ce biomatériau convient pour des applications potentielles de pansement.

En résumé, la stabilité et l'hydratation *in vitro* de la gélatine et du biomatériau composite ont été bien caractérisées. Ce nouveau biomatériau composite de RDDC peut être ultérieurement développé et exploité à des fins de production de pansements pour le traitement de blessures sur le champ de bataille.

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1. Introduction

DRDC Toronto has been developing a novel biomaterial for use as a wound dressing for the treatment of contaminated, painful, hemorrhagic battlefield wounds. The biomaterial can be best described as a hydrogel-elastomer interpenetrating polymer network (IPN). IPNs are best described as a combination of two crosslinked polymers, at least one of them synthesized or crosslinked in the immediate presence of the other [1]. IPNs have been extensively investigated to combine properties of a variety of materials. We used the IPN technique to combine two types of biomaterials: hydrogels and elastomers. It is expected that the hydrogel component could provide a moist wound cover conducive to healing; pain relief; exudate absorption; permeability to oxygen; and, protect the wound from infection. The elastomer component would confer desirable mechanical properties to the wound dressing as well as a barrier for the prevention of infection.

Gelatin, a biopolymer, was selected as the hydrogel component, and HydroThane™, a thermoplastic glycol-containing polyurethane, was chosen to form the elastomer component. In order to combine the two biomaterials to form an IPN, gelatin was modified through methacrylation to facilitate the crosslinkage by free radical polymerization. A variety of commercially available polyurethanes were examined and only HydroThane™ (CadioTech International Inc., City Woburn, MA) was found to be able to form an IPN with gelatin, by simultaneously crosslinking each polymer in solution [2]. To our knowledge, the formation of a hydrogel-elastomer IPN has never been reported.

Gelatin, extensively used in the biomedical [3], pharmaceutical [4, 5] and food industries [5], is bioactive and has been reported to be hemostatic [6]. Typically, gelatin can be further chemically or physically treated to obtain the desired properties for a variety of applications. For example, gelatin has been crosslinked by genipin [7]; complexed with xanthan gum [8]; interpenetrated with poly(2-hydroxyethylmethacrylate) [9]; modified with fatty acid esters [10]; and, blended with poly(vinyl alcohol) [11]. In yet another biomaterial, gelatin was combined with glucan and crosslinked by 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride to form a stratified wound dressing [12]. One drawback with this hydrogel dressing, however, was its poor mechanical strength.

Polyurethane is a synthetic polymer that can be made by condensation polymerization of polyols, diisocyanates and chain extenders (e.g., small molecules with either hydroxyl or amine end groups) [13]. The copolymer is a very attractive biomaterial because of its biocompatibility and unique mechanical properties [14]. However, the synthetic material is normally hydrophobic and cannot absorb a large amount of fluid, as required in some wound-dressing applications. In this study, we chose HydroThane™ because it is the only polyurethane that can co-dissolve in the same solvent (i.e., dimethylsulfoxide) for gelatin, the hydrophilic component of our IPN. The same polyurethane has also been studied for potential biomedical applications and is UV-curable using photo-initiators [15, 16].

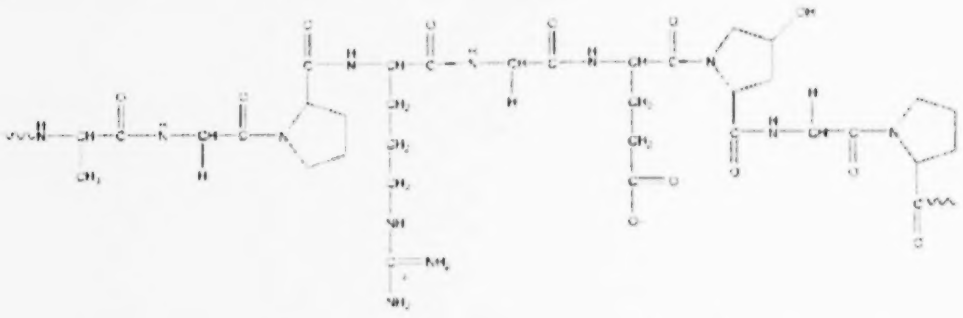
Swelling, degradation and/or dissolution of a biomaterial are important design parameters that can significantly affect their desired functions [17]. Depending on the application, the biomaterial may be required to show different degree of swelling and to be degradable or

soluble at different rates or even non-degradable. In our application, stable swelling and good absorbency are required for a wound dressing to treat highly exudating wounds. In addition, good mechanical properties are also important for the biomaterial to function properly. For example, elasticity is a desirable property for a wound dressing to conform to irregular surfaces [18].

This technical report describes a series of stability and hydration studies of gelatin and gelatin-HydroThane™ IPN composite biomaterials. The successful preparation and characterization of the IPN biomaterials should enable us to further develop this novel biomaterial for wound care on the battlefield.

2. Stability of gelatin in aqueous solutions

Gelatin is a biopolymer containing a typical repeating amino acid sequence of Gly-X-Y; where X and Y being mostly proline and hydroxyproline [19] (Fig. 1). It is derived from acidic or basic treatment of a collagen, a most abundant and ubiquitous protein in vertebrates [20]. There are two types of gelatin: Type A, derived from acid-processed materials, primarily pork skin; and Type B, derived from alkaline- or lime-processed materials, primarily cattle or calf hides and ossein (bone collagen).



Ala Gly Pro Arg Gly Glu Hyp Gly Pro

Figure 1. Chemical structure of gelatin.

A number of studies on the degradation of crosslinked gelatin have been reported [21-23]. In contrast, the information on the degradation of native gelatin is limited. It has been reported that both hydrolysis and enzymatic digestion may be involved in gelatin degradation [24]. Studies have been conducted towards controlling the degradation of gelatin-based biomaterials for a number of applications. For example, the hydrolysis of gelatin was examined for the controlled release of drugs [25, 26]. The effects of physical and chemical crosslinking on the enzymatic degradation of gelatin networks have also been explored [27]. In our work, gelatin was modified through methacrylation, then crosslinked in the presence of HydroThane™. The methacrylation did not involve hydrolyzable peptide bonds in the gelatin, but the crosslinking of gelatin in the IPN should increase its stability [27]. Therefore, the gelatin component in the IPN can still be degradable, but likely with a slower rate, due to the chemical crosslinking. In addition, physical crosslinking of gelatin through interpenetration with HydroThane™ may also increase gelatin stability [27].

To determine the stability of the gelatin-HydroThane™ composite biomaterial, the stability of native gelatin was investigated in aqueous solutions, where only hydrolysis would take place, and then the biomaterial's stability in the aqueous and serum-containing media was studied.

In this study, we used commercially available gelatins, which are heterogeneous mixture of polypeptides. Only a bloom number (a measure of stiffness of a gelatin hydrogel) is normally provided by the manufacturer. Therefore, it is necessary to estimate the molecular

weight and distribution as these are considered to have more impact on properties of gelatin such as gelation, processability and emulsion stability [28, 29]. For example, a high proportion of low- molecular-weight gelatin was found to result in low viscosity, low gelation temperature and long gelation time [30]. The methacrylation process itself may affect the molecular weight and molecular weight distribution pf gelatin, subsequently affecting the property of the composite biomaterial. It has been reported that the conditions in gelatin processing can affect its foam formation ability and stability, water-holding capacity and gel strength [31]. Therefore, it is important to develop an analytical tool to characterize gelatin degradation and to determine the molecular weights and molecular weight distribution of gelatin-based biomaterials.

Gel permeation chromatography (GPC) is a powerful tool for the study of both synthetic and natural polymers based on the determination of their molecular weights and distributions [32]. Both hydrolytic and enzymatic degradation of polymers have been characterized by GPC [33, 34]. Although polyacrylamide gel electrophoresis has been widely used for the molecular weight determination of proteins, including gelatin [35], it is not a quantitative method for molecular weight distribution.

In this section, a GPC method was validated to quantify the molecular weight and distribution of gelatin and then used to analyze their changes, as a gelatin aqueous solution was incubated at various temperatures for different periods of time. More specifically, polymer standards with known molecular weights and gelatin solutions with known concentrations were both characterized by the GPC. Two studies of gelatin stability were then undertaken: a short-term study involving the incubation of gelatin solution in distilled water at 50, 70 and 100°C for up to 4 h, and a long-term study involving incubation of gelatin solution in distilled water, supplemented with 0.1% sodium azide to prevent bacterial growth, at room temperature and 37°C for up to 18 days. The former was to confirm that within a short period of time (up to 4 h), the changes of the molecular weight and molecular weight distribution of gelatin in distilled water could be analyzed by the GPC method as a function of time. The latter study was designed to assess whether these changes were related to the stability of our IPN biomaterials in various media.

2.1 Validation of a gel permeation chromatography method

A GPC system was set up to determine the gelatin stability of biomaterials that we developed. The system was composed of PL aquagel-OH 30 and 40 GPC columns (Polymer Laboratories Inc., Amherst, MA) connected in series; a Waters 2690 separation module; and, a 996 UV detector (Waters Ltd., Milford, MA), all run by Millennium software. The running conditions and sample preparations were established, based on a method previously reported [36]. The mobile phase (1.8% sodium dodecyl sulphate in Milli-Q water) was filtered through 0.45- μm filters prior to its use. A 20-μL aqueous gelatin sample was injected into the GPC system and run for 20 min at a flow rate of 1 mL/min. The temperature of the sample and column compartments was set at 37°C. The UV detection was carried out at 220 nm to account for amide-bond absorption.

Poly(sodium styrenesulfonate) standards (Sigma-Aldrich, Mississauga, ON) with monodispersity less than 1.2 and with molecular weights over the range of 210-350,000 g/mol, were used to construct a calibration curve to calculate molecular weights of gelatin samples [36]. Figure 2 shows a typical calibration curve plotted as the peak molecular weight of the standards versus their retention time. A polynomial regression (R^2 =0.999) was fitted to the molecular weights over the range of 6,430-200,000 g/mol. Some deviations were observed for the standards with a molecular weight above or below this range, due to possible interactions of the standards with the column. The equation shown in Figure 2 was used to calculate the molecular weights of any unknown samples. This range covers the molecular weights of most interest (i.e., 10,000 to 200,000 g/mol) and it should be noted that the peak molecular weights of gelatin and its degradation products reported in this study fell into this range.

In addition to the standard curve for molecular weight characterization, another standard curve was required to quantify the amount of gelatin residuals, formed by the degradation of native and crosslinked gelatin in solutions, incubated at various temperatures for different time intervals. Briefly, standard solutions of gelatin (0.05, 0.2, 0.5 and 1 mg/mL) were prepared in distilled water supplemented with 0.1% sodium azide and analyzed using the GPC method described previously (Fig. 3). The area under each peak was then integrated using the instrument software. The standard curve shown in Figure 4 was obtained by plotting the concentration of the gelatin standard solutions against the total integration area under the corresponding gel permeation chromatogram of the standards, assuming that the UV absorption coefficient of the residuals at 220 nm was solely due to that of gelatin.

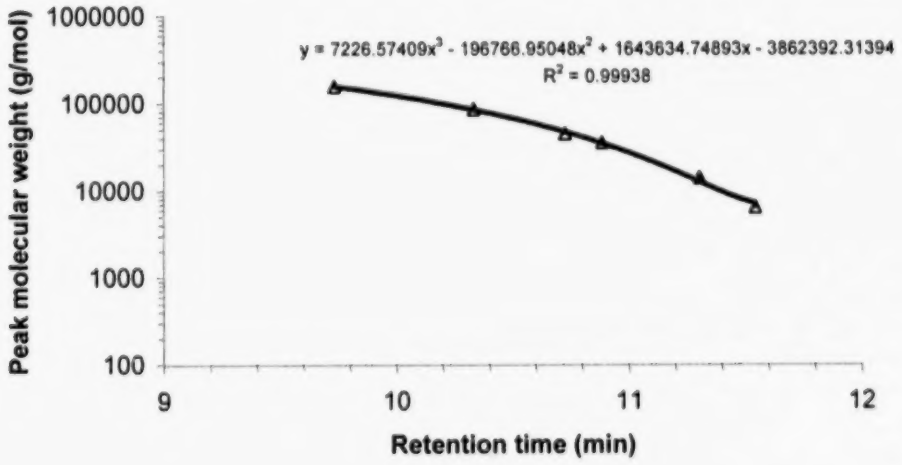


Figure 2. Typical calibration curve describing the relationship between the peak molecular weight of poly(sodium styrenesulfonate)s standards and their retention time. Data are expressed as means ± standard deviation (n=3).

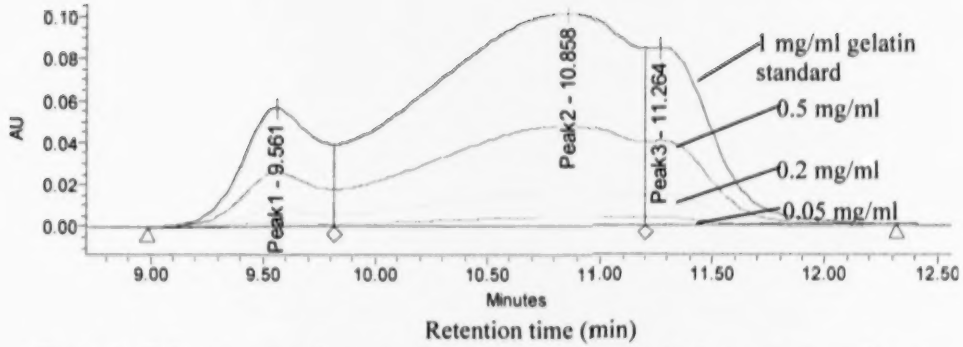


Figure 3. Gel permeation chromatograms of various standard gelatin aqueous solutions supplemented with 0.1% sodium azide. [AU] UV absorbance at 220 nm.

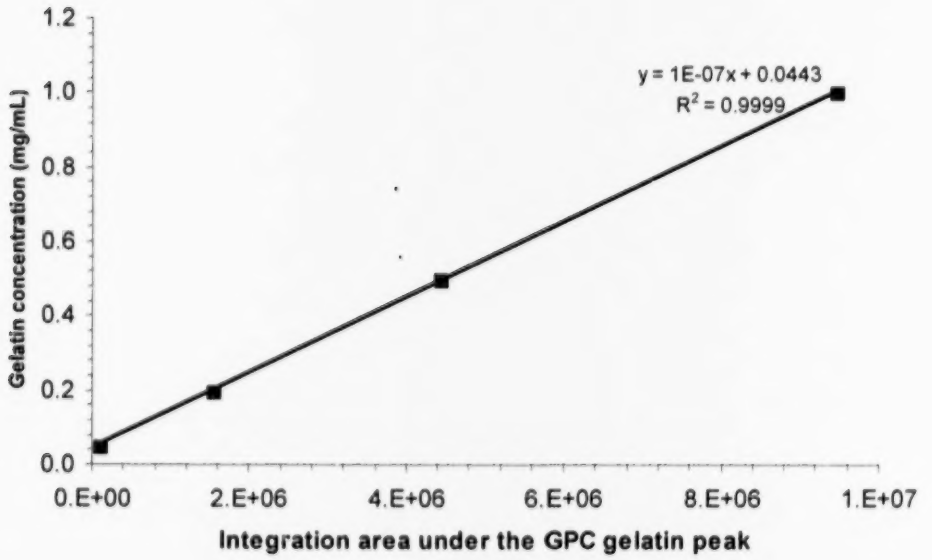


Figure 4. Typical standard curve describing the relationship between the concentration of different gelatin standard solutions and the integration area under the GPC gelatin peaks.

2.2 GPC study of native gelatin stability

To provide an indication of the extent of hydrolysis of native gelatin, the *in vitro* short-term (up to 4 h) and long-term (up to 3 weeks) stabilities of native gelatin were assessed in aqueous solutions.

2.2.1 Short-term stability study

A gelatin solution (1 mg/ml) was prepared in distilled water using Type A, Bloom 235 gelatin (Great Lakes Gelatin Inc., Grayslake, IL), and aliquoted (~10 mL) in scintillation vials. The latter were then sealed under nitrogen to prevent oxidation, and heated at 50°C, 70°C and 100°C in an oven for 4 h (n=3 per group except for 50°C where n=3). While 50°C was selected because this temperature is currently used for the methacrylation of the gelatin [2], assessment at 70°C and 100°C was also carried out to confirm our hypothesis that the hydrolysis of gelatin is temperature-dependent. At regular time intervals, 0.5 mL aliquots were taken from each vial for GPC analysis as detailed in Section 2.1, to assess changes in gelatin molecular weight and distribution.

Figure 5 illustrates typical gel permeation chromatograms obtained during incubation of the gelatin solution for up to 4 h at 100°C. Three peaks with retention times of 9.562, 10.822 and 11.252 min were identified, representing three different molecular weight fractions of gelatin corresponding to γ, β, α chains in the macromolecule [37]. Using the GPC instrument software, the chromatogram generated was accordingly subdivided into molecular-weight ranges. This enabled a determination of the molecular weight fractions that may undergo different hydrolysis. The area under the peak 1 at 9.562 min as indicated in red corresponds to the high-molecular-weight fraction of gelatin (i.e., $HiMw_{gelatin}$) [37]. The area under peaks 2 and 3 as indicated in green is related to the low-molecular-weight fraction of gelatin (i.e., $LoMw_{gelatin}$) [37]. As shown in Figure 5, the $HiMw_{gelatin}$ decreased with time and disappeared as gelatin was heated at 100°C for 4 h. The corresponding molecular weight at each peak was calculated for gelatins, incubated in water at different temperatures for different time intervals, using the calibration curve shown in Figure 2.

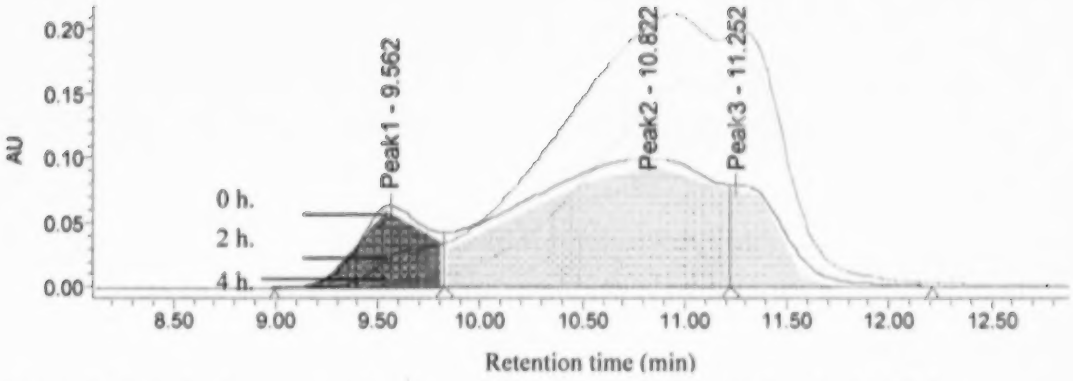


Figure 5. Typical gel permeation chromatograms of gelatin solutions incubated at 100°C for 0, 2 and 4 h. AU UV absorbance at 220 nm. Peaks 1, 2 and 3 represent γ, β, α chains, respectively.

Figure 6 shows the effects of temperature and duration of incubation on the molecular weight of the high-molecular-weight fraction of gelatin. It is clear that the 4-h incubation is insufficient to observe degradation in the gelatin at 50°C. In contrast, a reduction in the molecular weight of the $HiMw_{gelatin}$ was very evident in the samples heated to 70°C. Moreover, the $HiMw_{gelatin}$ disappeared after a 4-h incubation at 100°C. The $LoMw_{gelatin}$ showed a similar decrease in molecular weights at the three incubation temperatures (Fig. 7). However, the effects of temperature and duration of incubation on the $HiMw_{gelatin}$ appeared more significant than those observed on the $LoMw_{gelatin}$.

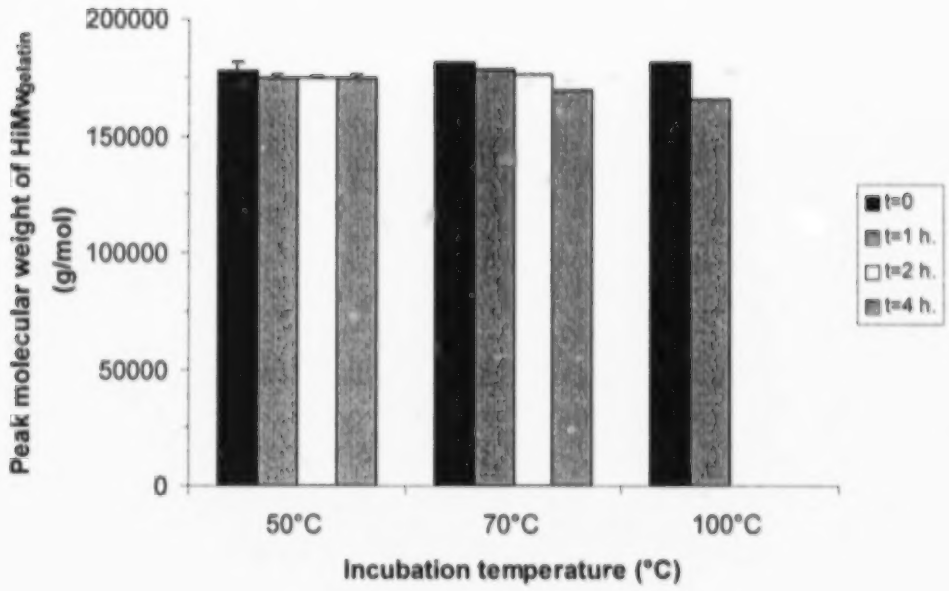


Figure 6. Effect of incubation temperature and period on the molecular weight of the high-molecular-weight fraction of gelatin ($HiMw_{gelatin}$) in aqueous solutions (1 mg/mL). Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

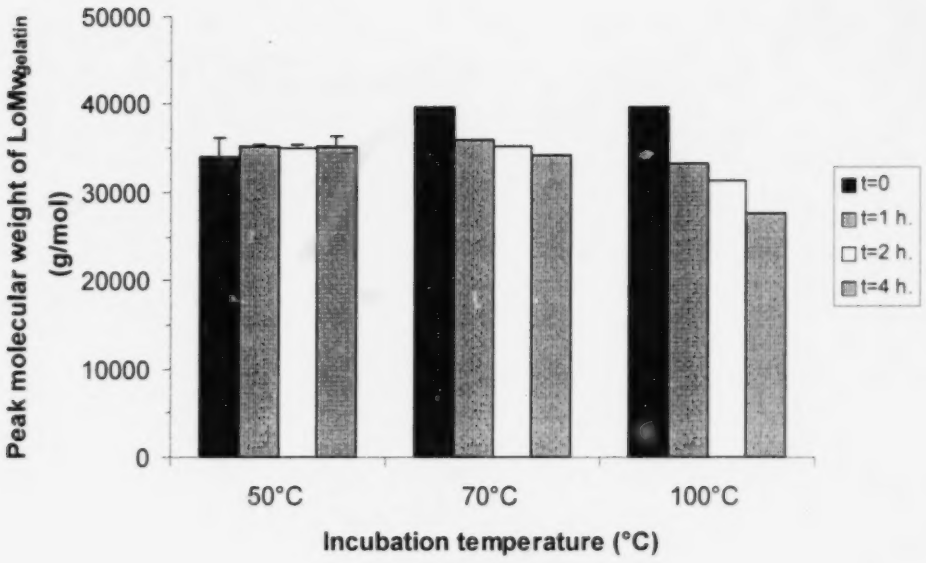


Figure 7. Effect of incubation temperature and period on the molecular weight of the low-molecular-weight fraction of gelatin ($LoMw_{gelatin}$) in aqueous solutions (1 mg/mL). Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

A common way to characterize the molecular-weight distribution of a polymer is to use the ratio between weight-average molecular weight and number-average molecular weight, known as polydispersity [38]. Our instrument software (i.e., Millennium version 3.2 by Waters Ltd.) was not designed for GPC analysis and could not calculate the average molecular weights of a polymer. Therefore, the ratio between the area under peak 1 and the total area under peaks 2 and 3 (i.e. the weight ratio between the $HiMw_{gelatin}$ and the $LoMw_{gelatin}$) was designated as $HiMw_{gelatin}/LoMw_{gelatin}$ ratio and calculated to indicate the molecular-weight distribution.

Figure 8 shows the changes in the composition (i.e., $HiMw_{gelatin}/LoMw_{gelatin}$ ratio) of the various gelatin solutions as a function of the incubation period or the incubation temperature. There was a decrease in the amount of the $HiMw_{gelatin}$, which became more significant with increasing temperature and time. Specifically, no statistically significant changes took place at 50°C. A decrease of about 75% in the $HiMw_{gelatin}$ was observed after a 4-h incubation at 70°C. The decrease was so fast at 100°C that no $HiMw_{gelatin}$ could be identified in the GPC chromatogram after the 4-h incubation.

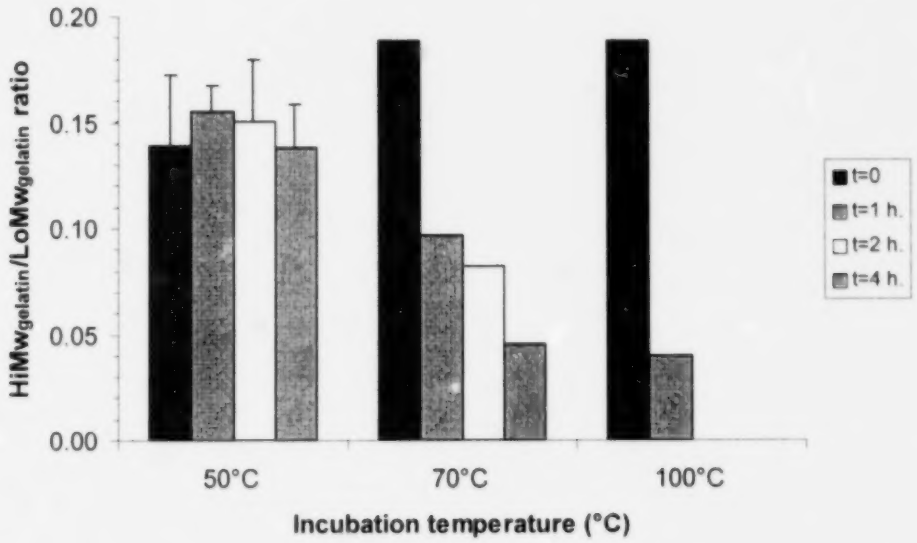


Figure 8. Effect of incubation temperature and period on the molecular weight distribution of gelatin in aqueous solutions (1 mg/mL). Data with error bars represent means ± standard deviation (n=3), otherwise n=1. $^{LoMw\ gelatin}$ Low-molecular-weight fraction of gelatin. $^{HiMw\ gelatin}$ High-molecular- weight fraction of gelatin.

In summary, the GPC analysis enabled us to characterize the molecular weight and molecular-weight distribution of gelatin. Our finding shows that the gelatin used for the preparation of the DRDC biomaterial contains mixed fractions, with a range of molecular weights. Our observation is consistent with reported molecular-weight heterogeneity of gelatin [39]. The incubation temperature and period both affected the molecular weight and molecular-weight distribution of gelatin. It seems that the $HiMw_{gelatin}$ was more affected by these parameters and degraded to lower molecular weights, leading to a corresponding increase in the amount of the $LoMw_{gelatin}$. Gelatin with different bloom numbers may contain different amounts of each fraction, and the higher-bloom-number gelatin may contain a larger amount of the $HiMw_{gelatin}$ [39], which is more susceptible to hydrolysis.

2.2.2 Long-term stability study

To study the long-term stability of gelatin in solution at room temperature and 37°C, gelatin solutions (1 mg/ml) were prepared as described in the previous section (n=3 per group), with the exception that they were supplemented with 0.1% sodium azide

to prevent bacterial growth. Aliquots of the solutions (~10 mL) remained at room temperature for up to 18 days, or were heated at 37°C in an oven for the duration of the experiment. It is noteworthy that these studies were conducted at room temperature to provide an estimate of the extent of gelatin loss in our gelatin-HydroThane™ IPN biomaterial under the experimental conditions used for the preparation and characterization of the films. Similarly, studies were carried out at 37°C to provide an indication of the gelatin loss that our material would likely sustain *in vivo*. Aliquots (0.5 mL) were taken at regular time intervals from each vial for GPC analysis (see Section 2.1) to assess the changes in both the molecular weight of gelatin and its distribution. It is noteworthy that the first sample taken for GPC analysis, referred to as "initial", was analyzed 2 hours after the gelatin was first mixed in the aqueous solution.

Figure 9 presents chromatograms of the gelatin solutions heated at 37°C for up to 18 days. Similar to the short-term study, the results show peaks corresponding to three different molecular-weight fractions of gelatin, associated with the γ, β, and α chains in the macromolecule. As discussed in Section 2.2.2, the area of peak 1 (i.e., γ chain) as indicated in red corresponds the $HiMw_{gelatin}$ and the area of peak 2 (i.e. β chain) and peak 3 (i.e., α chain) as indicated in green corresponds to the $LoMw_{gelatin}$. The molecular weight of each type of chains in the gelatin solution, after different periods of incubation, was calculated by replacing x in the equation shown in Figure 2 with the retention time for each peak.

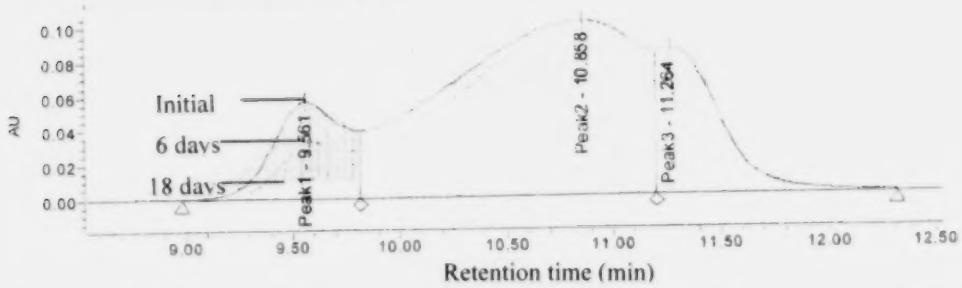


Figure 9. Gel permeation chromatograms of gelatin solutions (1 mg/mL) supplemented with 0.1% sodium azide and heated at 37°C for 2 h (initial), or for 6 or 18 days. AU UV absorbance at 220 nm. Peaks 1, 2 and 3 represent γ, β, α chains, respectively.

The quantitative results summarized in Figures 10, 11, 12 indicate the changes in the gelatin molecular weights and their distributions at 37°C and room temperature. A gradual decrease in the molecular weight of the γ chain with incubation time was observed at both 37°C and room temperature. Moreover, the reduction rate at 37°C is about 1.5 fold greater than that observed at room temperature. On the other hand, only slight decreases in the molecular weights of β and α chains were initially observed, becoming minimal after 6 days. The amounts of the $HiMw_{gelatin}$ and $LoMw_{gelatin}$ were calculated by replacing x in the equation shown in Figure 4 with the integration areas under peak 1 (i.e., γ chain) and peaks 2 and 3 (i.e., β and α chains).

The integration area was calculated using the instrument software. The changes in the composition were then calculated as $HiMw_{gelatin}/LoMw_{gelatin}$ ratio and related to changes in the molecular-weight distribution. Figure 12 shows a pattern of initial decreases, followed by a steady state after 6 days, irrespective of the incubation temperature of the gelatin solution, in the weight ratios between the $HiMw_{gelatin}$ and $LoMw_{gelatin}$. The initial decrease was faster at 37°C than at room temperature. The temperature-dependent hydrolysis is consistent with the short-term stability study showing increased changes in the composition with increasing incubation temperatures. The higher amount of the $HiMw_{gelatin}$ at 37°C than at room temperature at the beginning (i.e., day 0) may be due to an increase in solubility of gelatin at higher temperatures.

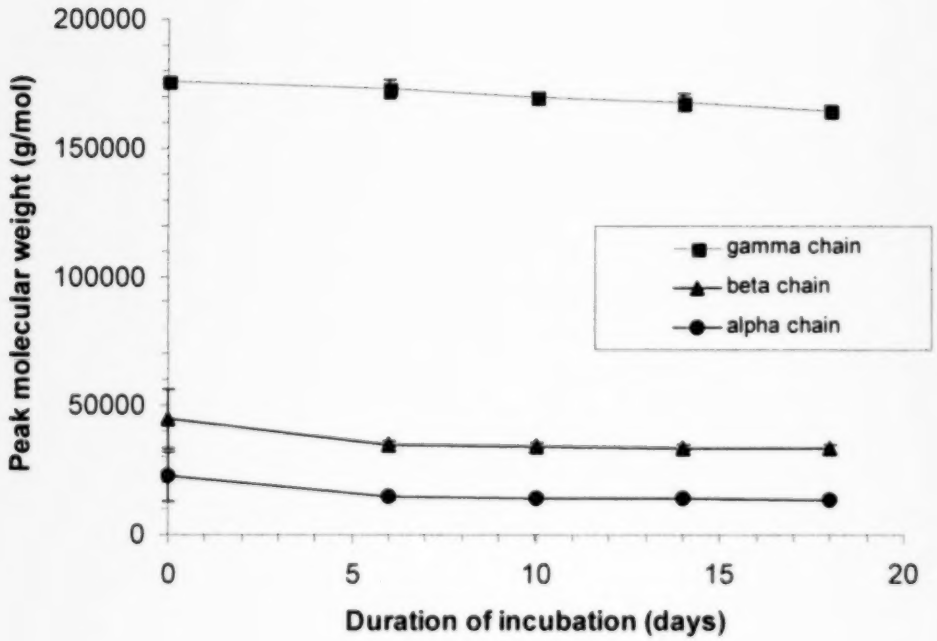


Figure 10. Changes in the peak molecular weight of each fraction of gelatin in a 0.1% sodium azide solution (1 mg/mL) maintained at 37 °C for 18 days. Data are expressed as means ± standard deviation (n=3).

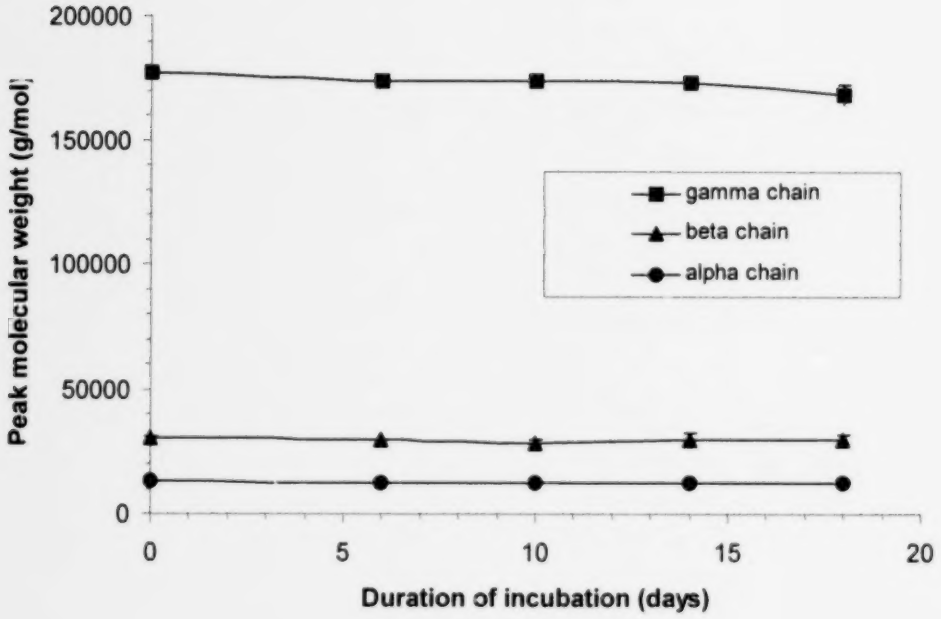


Figure 11. Changes in the peak molecular weight of each fraction of gelatin in a 0.1% sodium azide solution (1 mg/mL) at room temperature for 18 days. Data are expressed as means ± standard deviation (n=3).

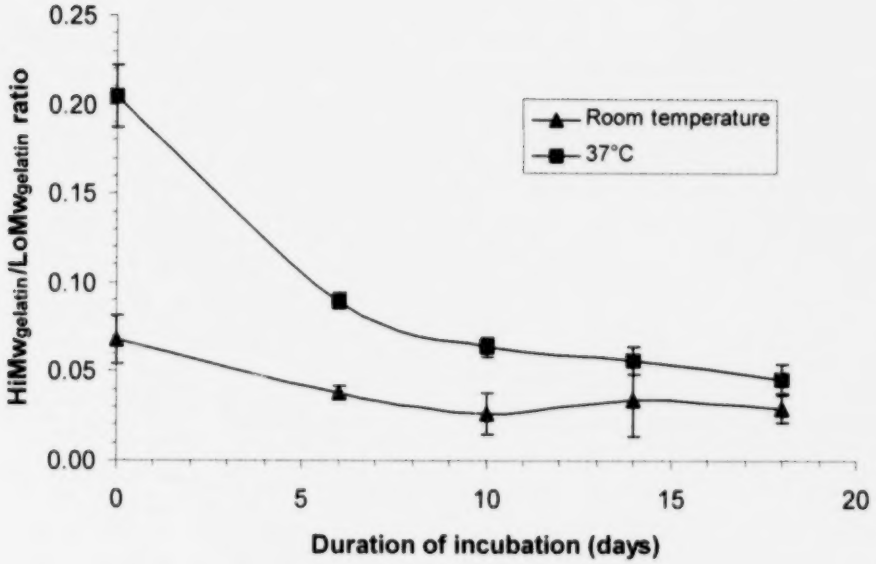


Figure 12. Changes in the molecular weight distribution of gelatin in a 0.1% sodium azide aqueous solution maintained at room temperature or heated at 37 ℃ for up to 18 days. Data are expressed as means ± standard deviation (n=3).

It is noteworthy that a gelatin solution, with a sufficiently high concentration (>5 mg/mL), is able to gel at a temperature below about 30°C, most likely due to the physical crosslinking of gelatin (i.e., formation of a helix structure) [40]. This would increase the stability of gelatin in the solution incubated at room temperature. Therefore, we prepared all the gelatin solutions at the concentration of 1 mg/mL, lower than the minimum gelation concentration, to avoid the effect of the physical crosslinking on the gelatin's stability at room temperature.

2.2.3 Recommendations for the development of the gelatin-HydroThane™ IPN

Both the short- and long-term stability studies show the temperature-dependent hydrolysis of gelatin in an aqueous solution. Changes in the high-molecular-weight (γ chain) fraction of gelatin appeared to be more significant than those occurring in the low-molecular-weight fraction. There is a large diversity in the chemical linkages in

gelatin. The cleavage of pentosidine and pyridinoline crosslinks as well as peptide bonds involving N-terminal proline, hydroxyproline and alanine has been attributed to gelatin hydrolysis [42]. Considering the fact that the γ chain is composed of three cross-linked α chains [41], our results strongly suggest that the degradation of gelatin over time is mediated by the hydrolysis of the intramolecular linkages in the γ chain, rather than any chemical bonds in the α chain.

As previously mentioned, gelatin was first modified for the preparation of our IPN biomaterials. The process involves the preparation of a gelatin aqueous solution at 50°C; methacrylation at the temperature for 1 h; and, finally membrane dialysis at room temperature or 37°C for a week. Its stability during the process may affect the properties of the modified gelatin. Furthermore, gelatin was incorporated into our IPN biomaterial as a highly absorbent component. Therefore, its stability in the biomaterial is important, in order to maintain the absorbency required for our intended wound care applications.

There are several implications of our stability study of gelatin for the development of our gelatin-HydroThane™ IPN biomaterial. First, a 1-h methacrylation of gelatin at 50°C should be conducted as soon as gelatin is completely dissolved to reduce gelatin hydrolysis. Second, the dialysis process after the methacrylation, in terms of time and temperature, should be controlled to reduce batch-to-batch variations. For example, methacrylated gelatin dialyzed at room temperature versus 37°C for a week might be subjected to different hydrolysis, leading to a difference in molecular weight and its distribution, as observed in the stability study at the two temperatures. Third, the temperature-dependent stability of gelatin may be correlated to its stability in the composite biomaterial in solution. Finally, our GPC studies clearly show that the gelatin used for the preparation of our gelatin-HydroThane™ IPN biomaterial contains mixed fractions of gelatin with a wide range of molecular weights. Considering our finding of a greater degradation of $HiMw_{gelatin}$ compared to $LoMw_{gelatin}$, one might speculate that gelatins with higher bloom numbers would yield larger amounts of $HiMw_{gelatin}$ and may exhibit more degradation.

It should be noted that in our application, the gelatin-containing dressing is designed to be placed in a wound for no more than 5 days, during which some gelatin degradation may occur, especially considering the presence of proteases at the wound site. We believe the results of the present study, examining the effects of gelatin degradation on the physical properties of our gelatin-HydroThane™ IPN, are valuable in providing guidance for us to design an IPN biomaterial for wound-dressing applications.

3. Stability of gelatin-HydroThane™ composite biomaterials in aqueous solutions

The stability of a biomaterial is determined by a number of factors including energy required to break the chemical bonds in the molecules; location of the bonds; the physical structure of the biomaterial; and, the environments to which the biomaterial is exposed (e.g., heat, chemicals and bio-catalysis). A biomaterial with strong covalent bonds in the backbone (e.g., C-C) is more stable than the one with hydrolysable groups (e.g., -CONH-, -COO- and C-O-C). The two polymers in our composite biomaterials (gelatin and HydroThane™) both contain hydrolysable bonds and may degrade through breakage of these bonds, thus potentially affecting their functions. A series of studies were designed to investigate the stability of the DRDC gelatin-HydroThane™ composite biomaterial, with an emphasis on the gelatin component, which was expected to be less stable than the HydroThane™ component.

The stability of a biomaterial in a solution is usually estimated by a gravimetric method [43]. Potential problems were identified in its application to this study. First, there was a risk that the biomaterial may break into tiny pieces due to repeated manipulations during the study. Isolating and weighing them repeatedly at different time intervals were impractical and inconvenient. Second, a large number of samples were required to facilitate the repeated withdrawal of samples for dry weight determination at various periods. Third, there was a further risk that the solutes (e.g., sodium azide) from the immersion solution may be absorbed by the sample resulting in an increase in its dry weight. This may become significant when the solution contains a solute at a high concentration. Alternatively, spectrophotometric methods have been used to quantify the degradation of gelatin composites using infrared [23] and UV-visible spectroscopy [24].

To overcome some of the drawbacks of the gravimetric method, we selected gel permeation chromatography (GPC). GPC has been used to study the stability of different types of biomaterials including hydrogels [44] and elastomers [45]. It provides information for both degradation rates and mechanisms of degradation [46]. Moreover, dissolution and degradation effects on the stability of biomaterials can be distinguished by analyzing the molecular weights of solutes in the solution, in which the biomaterial has been immersed. Therefore, the GPC method described in Section 2 was used to study the stability of our composite biomaterial.

3.1 Preparation of gelatin-HydroThane™ IPN films

Gelatin (Type A, Bloom 235) purchased from Great Lakes Gelatin Inc. (Grayslake, IL) was methacrylated according to a modified procedure [47]. Specifically, a 10-g sample of gelatin was added to 100 mL phosphate buffered saline (PBS, pH 7.4) and stirred at 50°C until complete dissolution. A 0.5 or 1-mL aliquot of methacrylic anhydride (MAAH; Aldrich, Mississauga, ON) was added to the gelatin solution, the latter step being performed under nitrogen in a glove box to prevent hydrolysis of methacrylic anhydride to methacrylic acid.

The reaction mixture was stirred for 60 min at approximately 50°C, and dialyzed against distilled water, at elevated temperatures around 37°C for 1 week, before freeze-drying to constant weight. Dialysis membranes with a molecular weight cut-off of 12,000-14,000 (Fisher Scientific, Nepean, ON) were used.

IPN films were prepared by *in situ* photo crosslinking of methacrylated gelatin and HydroThane™ in a solution, containing an equal amount of each polymer and a photo-initiator, 2, 2-dimethoxy-2-phenylacetophenone (Irgacure 651). More specifically, a 0.67 g 7.5 wt% methacrylated gelatin in dimethylsulfoxide (DMSO) was mixed with 1.25 g 4 wt% HydroThane™ in DMSO in a glass vial. Then 91-µl 10 wt% Irgacure 651 (Ciba Specialty Chemicals Canada, Toronto, ON) in DMSO was added. This pre-IPN solution was vigorously vortexed, flushed with nitrogen for 5 min and UV irradiated for 15 min at 350 nm at an intensity of 9 mW/cm^2 by a photochemical chamber reactor (RAYONET model RPR-200, Southern New England Company, CT). The resulting films were washed in 0.1% sodium azide aqueous solution at ambient temperature for a week to remove DMSO and then freeze-dried until constant weight was reached. Each film had a diameter of 2.5 cm and thickness of 0.2 cm. Gelatin and HydroThane™ films were prepared, using the same methodology as for the preparation of the IPN films, and used as controls.

3.2 Stability of the gelatin component in the IPN films

The freeze-dried films (gelatin, HydroThane™ and gelatin-HydroThane™ IPN; n=2 per experimental group) were immersed in a fresh 0.1% sodium azide aqueous solution (25 mL) at 37°C and room temperature. At specific time intervals, a 1-mL aliquot of the solution was collected and replaced with 1 mL of a fresh 0.1% sodium azide aqueous solution. This solution was analyzed using the GPC method as described in Section 2.1 to gain a better understanding of the release kinetics of gelatin and HydroThane™ from the IPN films, as well as the mechanism underlying their degradation. The concentration and the amount of gelatin residuals in the solution were calculated at each time interval from the entire area under the chromatogram using the standard curve shown in Figure 4. Gelatin loss was calculated as a percentage of the cumulative amount of the degradation products of gelatin in the solution over the initial amount of gelatin in the film.

Figure 13 shows typical gel permeation chromatograms of the aqueous solution in which the photo-crosslinked gelatin and IPN films were immersed at room temperature and 37°C for up to 6 days. Lower amounts of gelatin residuals were detected at room temperature than at 37°C for both IPN and gelatin films over the immersion period. Furthermore, HydroThane™ was very stable, as suggested by the lack of residuals in the solution in which photo-crosslinked HydroThane™ films were incubated for 34 days. In fact, less than 1% of the HydroThane™ was recovered from the incubation medium over a 2-month immersion period in 0.1% sodium azide solution at 37°C (Fig. 15).

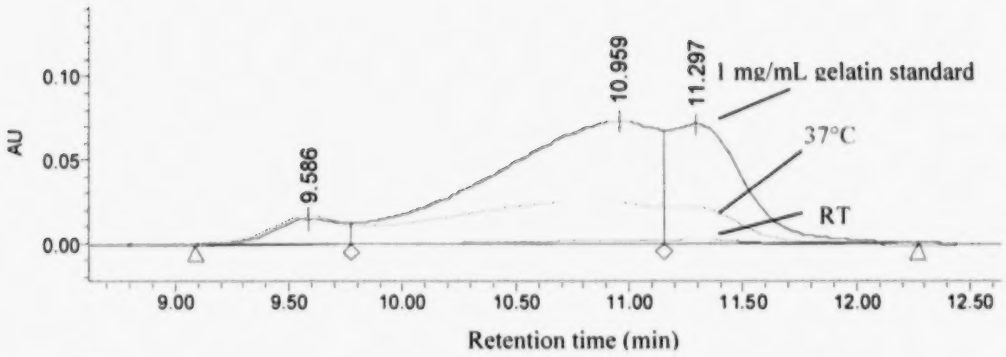


Figure 13. Gel permeation chromatograms of the 0.1% sodium azide aqueous solutions in which photo cross-linked gelatin films were immersed at 37 ℃ or room temperature (RT) for 5 days. [AU] UV absorbance at 220 nm.

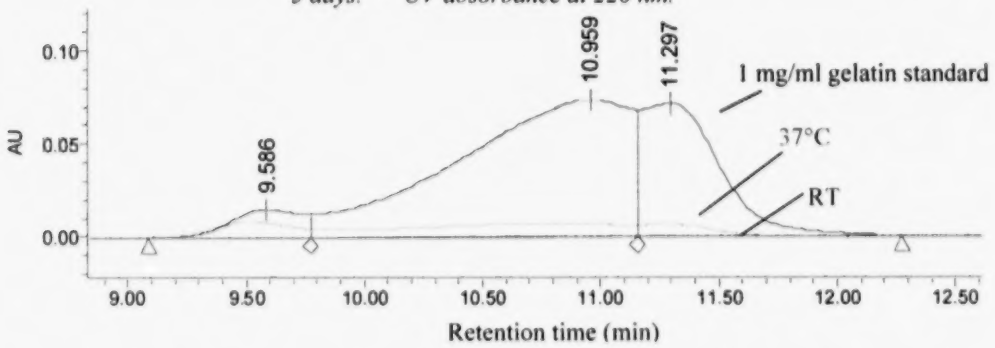


Figure 14. Gel permeation chromatograms of the 0.1% sodium azide aqueous solutions in which photo cross-linked gelatin-HydroThane™ films were immersed at 37 ℃ or room temperature (RT) for 6 days. [AU] UV absorbance at 220 nm.

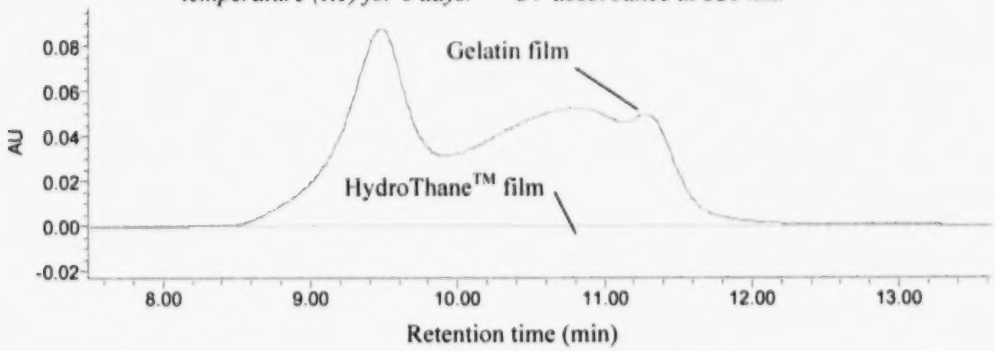


Figure 15. Gel permeation chromatograms of the 0.1% sodium azide aqueous solutions in which photo cross-linked gelatin or HydroThane™ films were immersed at 37 ℃ 34 days. [AU] UV absorbance at 220 nm.

Figure 16 shows an increasing loss of gelatin over time for both freeze-dried IPN and gelatin films immersed in a 0.1% sodium azide aqueous solution. The loss of gelatin from the gelatin film at 37°C was initially dramatic, and then slowed down, reflecting the kinetics shown in Figure 12, this loss being greater at 37°C than at room temperature. More specifically, about 30% and 15% of gelatin in the crosslinked gelatin and IPN films were degraded after a 10-day immersion at 37°C. The corresponding values were about 5% for both types of films at room temperature. In addition, gelatin in the IPN films was more stable and sustained than that in the gelatin films, especially at the beginning of 20 days, due to its entanglement with HydroThane™ in the IPN. The gelatin loss was up to 50% after immersion of the IPN film for 34 days at 37°C. The release of the gelatin component from our biomaterials may compromise its functions as a hydrogel for sustained drug release and water absorbency, and affect its biological response. In contrast, less than 1% weight loss of HydroThane™ was observed even after a 2-month immersion in the 0.1% sodium azide solution at 37°C.

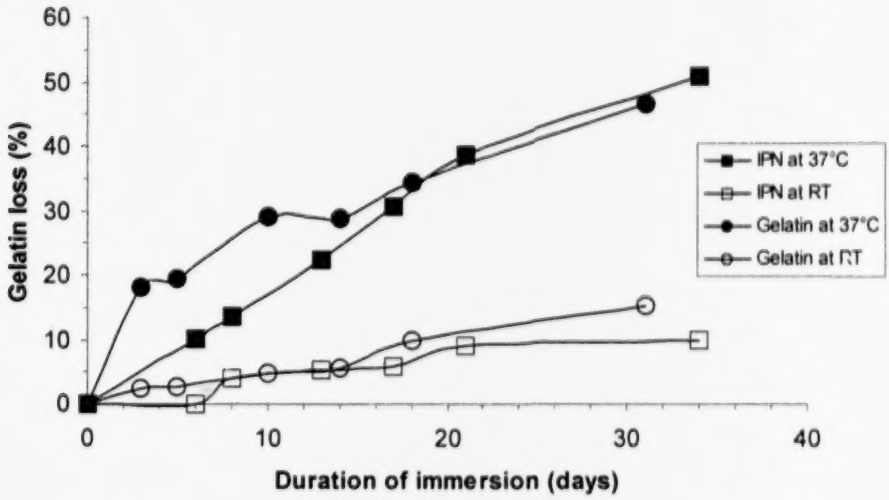


Figure 16. Stability through gelatin loss of freeze-dried gelatin-HydroThane™ Interpenetrating Polymer Network (IPN) films and crosslinked gelatin films, immersed in a 0.1% sodium azide solution at 37 °C and room temperature (RT) for up to 34 days. Data are expressed as means (n=2).

To better understand the mechanism of the gelatin loss from the photo cross-linked gelatin films and gelatin-HydroThane™ films during a long-term immersion in aqueous solutions, the change in gelatin composition was determined. A small reduction in the ratio between $HiMw_{gelatin}$ and $LoMw_{gelatin}$ was observed during the first 10 days of immersion at room temperature, irrespective of the type of films under study. It is noteworthy that this change in gelatin composition of the photo cross-linked films at room temperature mirrors that observed for native gelatin solutions (Fig. 12). In contrast, a marked increase in the ratio between

$HiMw_{gelatin}$ and $LoMw_{gelatin}$ in the incubation medium was observed at 37°C during the 34-day study, this increase being greater after 4 days for the gelatin-HydroThane™ IPN than the gelatin films (Fig. 17). This differential change in the composition of native gelatin compared to those of the cross-linked gelatin or gelatin-HydroThane™ films might be related to the fact that cross-linking increased the molecular weight of gelatin degradation products, as represented schematically in Fig. 18. As a result, there was an increase in the high-molecular-weight fraction in the degradation products from cross-linked gelatin and IPN films. The discrepancies in gelatin composition at room temperature and 37°C might imply that different mechanisms are responsible for the observed gelatin loss from the photo cross-linked films. Indeed, while the changes observed at room temperature likely reflect solely a greater dissolution of the $LoMw_{gelatin}$ compared to that of the $HiMw_{gelatin}$ in both types of films, increasing the incubation temperature to 37°C caused a hydrolysis of the cross-linked gelatin, leading to a greater amount of $HiMw_{gelatin}$. This may imply that the effect of degradation products from gelatin in the film on biological response might be different from that of degradation products from native gelatin due to their different molecular weights and molecular weight distributions.

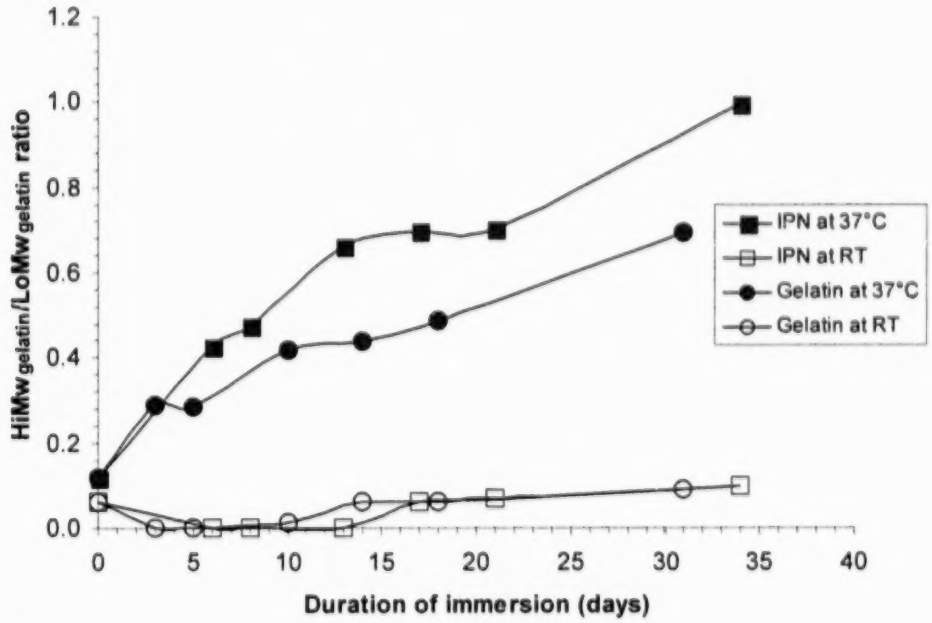


Figure 17. Changes in the molecular weight distributions of gelatin residuals released in the 0.1% sodium azide aqueous solution in which gelatin-HydroThane™ IPN films and crosslinked gelatin films were immersed at 37 °C and room temperature (RT) for up to 34 days. Data are expressed as means (n=2). $^{LoMw\ gelatin}$ Low-molecular-weight fraction of gelatin. $^{HiMw\ gelatin}$ High-molecular- weight fraction of gelatin.

Figure 18. Schematic illustration of an increase in the high-molecular-weight fraction of a crosslinked gelatin film immersed at 37 °C. The hydrolysable bonds between the polymer chains are indicated as "-". Hydrolysis leads to the formation of two branched polymer chains (i.e. an increase in the high-molecular-weight fraction of the degradation products).

3.3 Effects of preparation conditions on the hydration of IPN films

While our previous data clearly show that the gelatin component in our IPN is hydrolysed during immersion of the biomaterial in an aqueous solution, it remains unclear to what extent this degradation will affect its absorbency. However, it is generally acknowledged that the crosslinking density in gelatin-based biomaterials is a key factor for controlling swelling and degradation [4, 6]. Furthermore, IPN biomaterials are generally more stable if steps are taken to prevent phase-separation of their components during their preparation [1]. We therefore designed a series of experiments to assess effects of various factors on the hydration and morphology of the gelatin-HydroThane™ IPN films, in an attempt to further optimize our processing steps. More specifically, a number of conditions for the preparation of the IPN films were investigated to control the crosslinking density by modulating the degree of methacrylation and the use of additional crosslinkers. In addition, we assessed two different procedures for preventing phase separation of the gelatin and HydroThane™ components by aging the methacrylated gelatin solutions for different periods and freezing the pre-IPN solutions at different temperatures.

3.3.1 Extent of chemical crosslinking of gelatin

Gelatin was methacrylated using the same procedure as described in Section 3.1. In addition, different amounts of methacrylic anhydride were used with or without the addition of dimethylaminopyridine (DMAP) and triethylamine (TEA) to vary the degree of methacrylation [48]. Table 1 summarizes the conditions used in the methacrylation process and the extent of methacrylation. The extent of methacrylation was quantified by Cambridge Polymer Group Inc. using ^{1}HQS 400-1 Nuclear Magnet Resonance (NMR) with an internal maleic acid standard in

deuterated DMSO (DMSO-d_6, chemical shifts, δ, in ppm): 5.3~5.6 (=CH_2). The degree of methacrylation is expressed as a percentage of methacrylate groups over total ε-amino groups in gelatin and calculated using the equation:

$$Wt\%(=CH_2)/14 \Big/ 4.7/146.2 + 1.2/162.2$$

In this formula, wt% (=CH_2) is the weight percentage of =CH_2 group in methacrylated gelatin calculated from the relative intensity of =CH_2 proton and maleic acid proton, and 14 is the molecular weight of =CH_2. The numbers 146.2 and 162.2 represent the molecular weights of lysine and hydroxylysine with 4.7 and 1.2 wt% in gelatin, respectively, as specified by the manufacturer of the gelatin samples tested. The guanidino and hydroxyl groups were not included as they are not usually reactive with anhydrides in aqueous gelatin solution [49].

Table 1. Methacrylation of gelatin used for the preparation of gelatin-HydroThane™ IPN.

Methacrylated gelatin used (batch)	Methacrylic anhydride (mL)	Dialysis temperature (°C)	DMAP and TEA	Extent of methacrylation (%)
UNHEATED	0.5	Room temperature	No	9
HEATED	0.5	35-40	No	9
DMAP & TEA	0.5	35-40	Yes	18
2 MAAH	1	35-40	No	18

PBS Phosphate Buffered Saline DMAP Dimethylaminopyridine TEA Triethylamine

The same materials and methods, previously described in Section 3.1, were used to prepare gelatin-HydroThane™ IPN films (n=1 per experimental group) with different batches of methacrylated gelatin. IPNs were designated according to the gelatin source used for the IPN preparation as indicated in Table 1. In addition, a widely-used crosslinker was included in the film preparation to increase gelatin crosslinking. Specifically, N, N'-methylene-bis-acrylamide (Bio-Rad, Hercules, CA) was added at 20 wt% of methacrylated gelatin and then mixed with the pre-IPN solution prior to the UV irradiation under the conditions described in Section 3.1.

After a complete wash to remove DMSO, the films were then transferred in 25 mL of a fresh solution of 0.1% sodium azide, and immersed for up to 29 days at room temperature. The swelling ratio of the photo cross-linked IPN films, defined as the number of times the dry film absorbs its own weight in water, was calculated as the ratio of the wet weight over the initial dry weight at regular time intervals.

Figure 19 shows the hydration of the IPN films, prepared from different batches of methacrylated gelatin, as a function of time. Since gelatin crosslinking took place via the polymerization of methacrylate groups, gelatin with a higher degree of methacrylation would be more crosslinked and subsequently more stable. The

crosslink density has been reported to be proportional to the extent of methacrylation and thus gelatin with 9% methacrylation was likely slightly crosslinked [50]. It was confirmed by NMR analysis that the extent of methacrylation of gelatin used to prepare the DMAP & TEA and 2MAAH IPN films was twice that of gelatin used to prepare the UNHEATED and HEATED IPN films. As a result, gelatin in the latter IPN films was less crosslinked and released faster, leading to a decrease in the hydration of the films. This observation is consistent with the report on demonstrating reduced hydration stability with decreasing crosslinking density [51]. In addition, both UNHEATED and HEATED IPN films showed a decline in hydration after 10 days, suggesting that the stability of theses films was not affected by the dialysis temperature used in gelatin methacrylation. Although the extent of methacrylation of our samples was significantly lower than that reported by Schacht et al. [47] (9% versus 45%), the increase in methacrylation obtained by using a larger amount of methacrylic anhydride was consistent with their results.

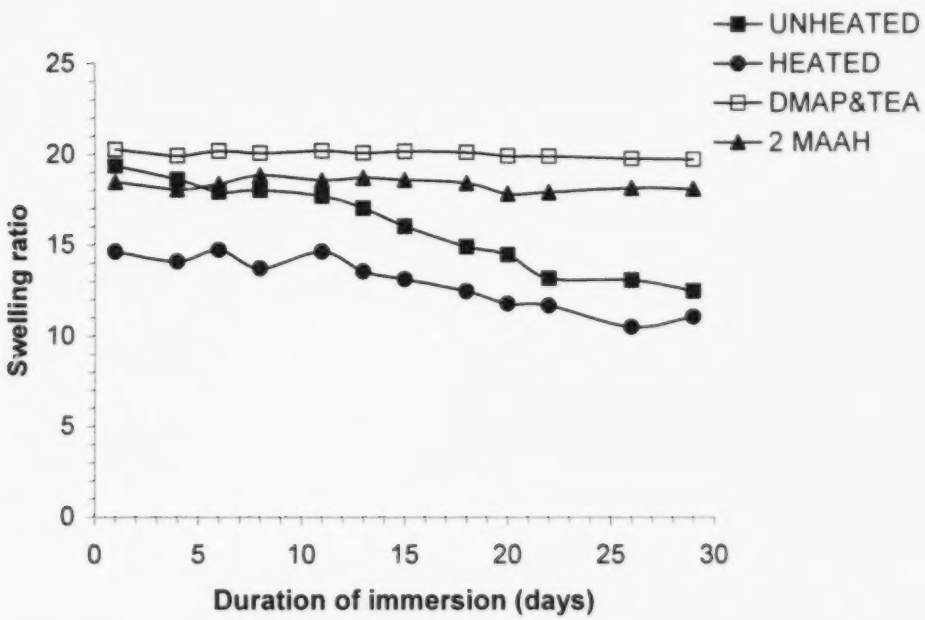


Figure 19. Changes in absorbency of gelatin-HydroThane™ films immersed in a 1% sodium azide aqueous solution at room temperature for up to 29 days. Films were prepared using various types of methacrylated gelatin (see details in Table 1). Data represent n=1 per time interval.

Swelling measurements have been widely used to characterize the crosslinking density of a hydrogel [52]. The swelling ratio has been inversely related to the crosslinking [52]. Furthermore, Flory's swelling equation can be used to calculate the

crosslinking density [53]. Based on the swelling ratio and crosslinking density reported for a chemically crosslinked gelatin swollen in an aqueous solution [54], we estimated that the crosslinking density of gelatin in our IPN films was below 4.1×10^{-4} moles/L, suggesting that our methacrylated gelatin was slightly crosslinked.

In addition to the extent of methacrylation of gelatin, the addition of an appropriate crosslinker to our pre-IPN solution would also increase the crosslinking degree. N, N'-methylene-bis-acrylamide is a widely-used crosslinker to make hydrogels through free radical polymerization [53]. Since the same reaction mechanism was involved in gelatin crosslinking to form the IPN film, it was expected that the addition of a crosslinker would increase the extent of crosslinking. As shown in Figure 20, the film prepared using the crosslinker appeared to have lower, but more stable hydration than the IPN prepared using the same batch of methacrylated gelatin without the crosslinker. On the other hand, considering our finding of a greater hydration of the film over the first 5 days of immersion, this period corresponding to the maximum period of application of the IPN as a wound dressing, we suggest that the addition of a crosslinker is not necessary.

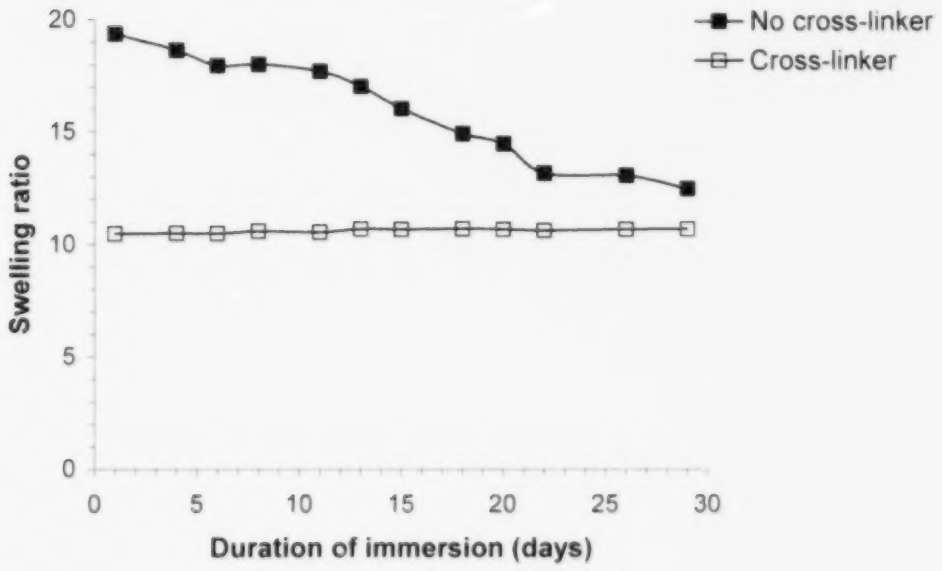


Figure 20. Changes in absorbency of gelatin-HydroThane™ IPN films immersed in a 1% sodium azide aqueous solution at room temperature for up to 29 days. Films were prepared with or without adding a cross-linker to the pre-IPN solution. Data represent n=1 per time interval.

3.3.2 Aging methacrylated gelatin solutions

IPN films were prepared with either a fresh methacrylated gelatin solution or solutions aged at 18 wt% for up to 3 weeks. Specifically, an 18 wt% methacrylated gelatin solution was prepared in DMSO and left in a sealed vial at room temperature for 1 to 3 weeks. The solution was then diluted to 7.5 wt% in DMSO, mixed with the HydroThane™ and Irgacure 651 solutions, and UV-irradiated under the conditions described in Section 3.1. The resulting gelatin-HydroThane™ IPN films were washed in a 0.1% sodium azide solution for a week and then immersed in 25 mL of a fresh 0.1% sodium azide solution. The hydration of the films was measured at different time intervals as previously described.

Photographs of the gelatin-HydroThane™ IPN films were taken using a Nikon CoolPix™ 880 digital camera (Nikon Corporation, Mississauga, ON, Canada) positioned over the eyepiece of an Olympus BH-2 optical microscope (Olympus, Brampton, ON, Canada), set at a 100x magnification to characterize the domain size of each component.

Figure 21 shows that the freshness of the methacrylated gelatin solutions used in the preparation of our IPNs can affect their swelling stability in the aqueous solution at room temperature. The IPN films prepared using aged methacrylated gelatin solutions showed a more stable swelling than those prepared from the same batch of fresh methacrylated gelatin solution. This is due to the presence of smaller domains (i.e. less phase separation between gelatin and HydroThane™) in the IPNs prepared with the aged methacrylated gelatin solutions, as confirmed by light microscopy imaging analysis (Fig. 21). It was hypothesized that the aging process increased the physical crosslinkage of gelatin [47], leading to a decrease in the phase separation and domain size. Our finding of an increased hydration stability of IPNs prepared with methacrylated gelatin solutions is in agreement with the report demonstrating that increasing the miscibility of components of IPNs will reduce their degradation [55].

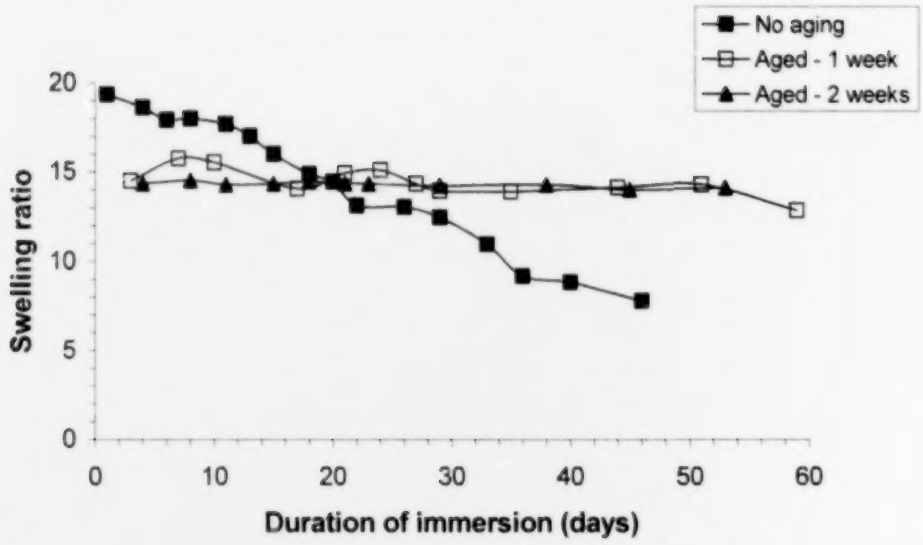


Figure 21. Changes in absorbency of gelatin-HydroThane™ IPN films immersed for up to 59 days in a 0.1% sodium azide aqueous solution maintained at room temperature. Films were prepared with either a fresh (no ageing) or an aged diluted methacrylated gelatin solution (aged), the latter solution being stored for one or two weeks at room temperature. Data represent n=1 per time interval.

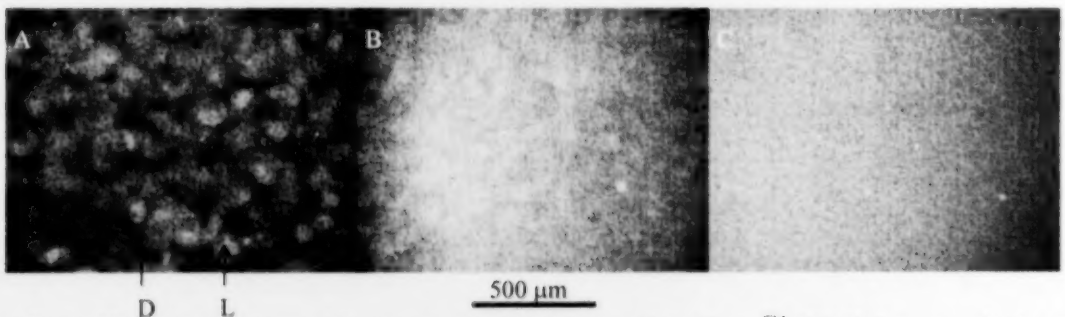


Figure 22. Light microscopy photographs of typical gelatin-HydroThane™ IPN films prepared using a fresh methacrylated gelatin solution (Panel A), or a solution stored at room temperature for one (Panel B) or two weeks (Panel C). The darker (D) and lighter areas (L) represent the HydroThane™ polymer and methacrylated gelatin, respectively.

Although aging methacrylated gelatin solutions appeared to maintain the swelling stability for more than 10 days, our results suggest that this procedure might not be an essential step for the preparation of the biomaterial as maximizing the absorbency of our IPN biomaterial is a critical factor. Indeed, the absorbency of the IPN prepared with the fresh methacrylated gelatin solution was significantly greater for the first 4 days of immersion in the aqueous solution (Fig. 21), this period corresponding to the standard duration of the washing procedure prior to freeze-drying the gelatin-HydroThane™ films.

3.3.3 Freezing the pre-IPN solutions

An alternative method for preventing phase separation of the gelatin and HydroThane™ components in the pre-IPN solution and during the IPN formation was developed. Briefly, the freezing method involved freezing the mixed pre-IPN solution containing methacrylated gelatin, HydroThane™ and Irgacure 651 at approximately 0, –70 or -200°C for at least 15 min, followed by the UV-irradiation and washing procedure as described in Section 3.1.

Figure 23 shows that the hydration of the films prepared using the freezing method tended to be lower but more stable than those prepared using the same batch of methacrylated gelatin without any freezing. We had hypothesized that the freezing method would prevent phase separation of the gelatin and HydroThane™ components, leading to the formation of smaller domains during IPN formation. This was confirmed by morphology characterization using light microscopy (Fig. 24) and attributed to an increased hydration stability. Our findings are consistent with those reporting reduced phase separation by quenching a pre-IPN mixture at difference temperatures [56].

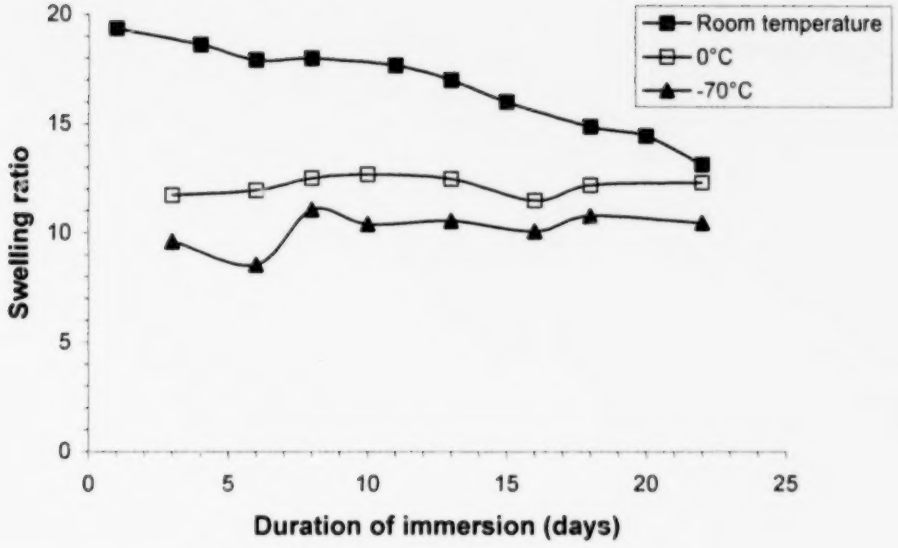


Figure 23. Changes in absorbency of gelatin-HydroThane™ IPN films immersed for up to 22 days in a 1% sodium azide aqueous solution maintained at room temperature. Films were prepared from pre-IPN solutions that were kept at various temperatures for up to 15 min prior to initiating the photo cross-linking process. Data represent n=1 per time interval.

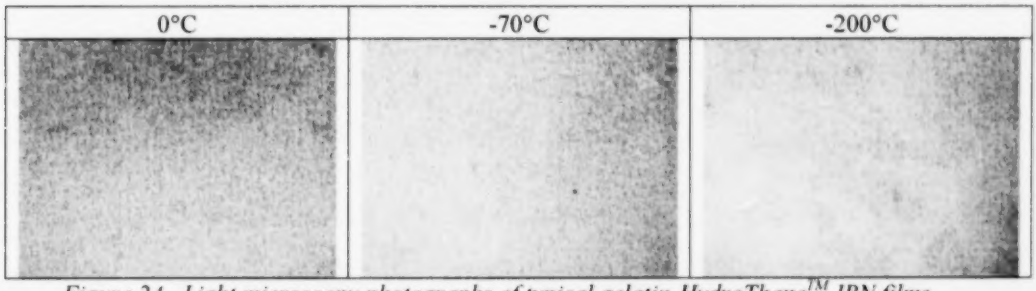


Figure 24. Light microscopy photographs of typical gelatin-HydroThane™ IPN films prepared using the freezing method. The pre-IPN solutions containing methacrylated gelatin, HydroThane™ and photo-initiator were frozen at 0 ℃, -70 ℃ or -200 ℃ for 15 min prior to UV-irradiation.

3.4 Re-hydration of freeze-dried gelatin-HydroThane™ IPN Films

Our results show that there is a degradation of gelatin in the IPN films immersed in an aqueous solution (Fig. 16). As gelatin is the component that confers absorbency to our wound dressing, it is important to determine to what extent this degradation would affect the hydration of our IPN biomaterials.

The extent of swelling of a biomaterial upon immersion in various media is commonly measured to characterize its water absorption properties as well as its degradation [57], although swelling and degradation of a biomaterial is not always directly related. Therefore, in addition to the GPC characterization, hydration studies were also conducted to confirm that the composite biomaterial could re-hydrate and maintain a level of hydration for our intended application. Specifically, photo cross-linked gelatin films and gelatin-HydroThane™ IPN films (n=3 per experimental group) were prepared and freeze-dried as described in Section 3.1, and immersed in 25 mL of a 0.1% sodium azide aqueous solution maintained at either at 37°C or room temperature. At pre-determined time intervals, the re-hydrated IPN films were removed from the solution, patted-dry, weighed and re-immersed in the medium. The hydration of each film was characterized by a swelling ratio as calculated according to the equation: Wt/Wo, where Wt and Wo represent the weight of the film at time t and 0 (initial dry film weight), respectively.

Figure 25 shows that the swelling ratios of the IPN film and gelatin control film were above 9. The extent of re-hydration of both types of films was greater at 37°C than at room temperature. On the other hand, the IPN films showed an average of a 30% lower re-hydration than their corresponding gelatin films, likely due to the fact that the presence of HydroThane™ in the IPN material was physically restricting the expansion of the biopolymer by creating a more rigid network. It is noteworthy that the re-hydration values of the IPN and gelatin films after a 34-day immersion remained constant, despite a significant gelatin loss was observed (i.e., 50% at 37°C; Fig. 16). This result may be explained by the fact that the degradation of gelatin in the IPN material led to the formation of pores that were then filled with the aqueous solution, therefore leading to the maintenance of the absorbency of the photo cross-linked films. Alternately, it is also possible that the loss of gelatin from the photo cross-linked films was responsible for loosening up their network structure, thereby leading to a greater hydration of the material [58].

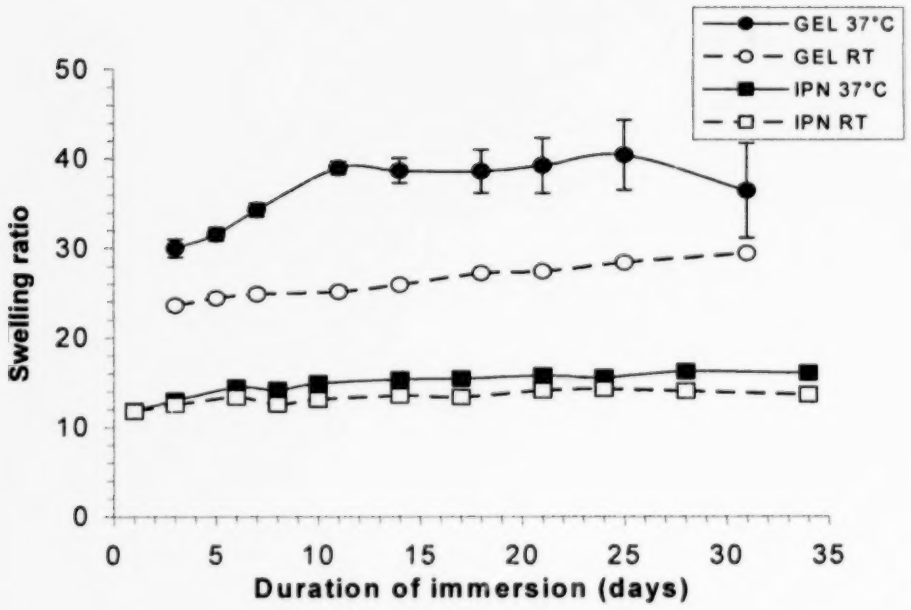


Figure 25. Re-hydration characteristics of freeze-dried gelatin-HydroThane™ IPN films and crosslinked gelatin (GEL) films immersed in a 0.1% sodium azide aqueous solution at 37°C and room temperature (RT) for up to 34 days. Data are expressed as means± standard deviation (n=3).

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4. Stability of gelatin-HydroThane™ composite biomaterials in serum

While the previous studies provided a better understanding of the stability of gelatin in films as well as identified the preparation conditions that may affect the absorbency of the gelatin-HydroThane™ IPN films, no inference can be made regarding the clinical relevance of our results. It is known that swelling and stability of a biomaterial are affected by the composition of the reacting solution as well as its pH, temperature, and the presence of biocatalysts and enzymes [59, 60]. A series of experiments were therefore conducted to examine the swelling of various types of gelatin-HydroThane ™ IPN films in diluted fetal bovine serum, in an attempt to simulate *in vivo* wound exudation. We used the swelling measurement (defined as the number of times the dry film absorbs its own weight in serum). Furthermore, we attempted to use the GPC method previously described in Section 2.1 to quantify the gelatin hydrolysis products in the diluted serum. However, serum components, likely to be albumin and globulins, overlapped with gelatin in the GPC chromatogram and thus, further separation between the serum proteins and gelatin is required to quantify gelatin stability in the IPN film. This is beyond the scope of the current study which is restricted to using the swelling measurement to characterize the stability of our biomaterial films.

The IPN films were prepared under the same conditions as described in Sections 3.1. In addition, a photo-initiator solution was prepared at different concentrations (5%, 10%, 20%) and added to the pre-IPN solution to study the effect of altering the concentration of this parameter. All films were freeze-dried for further characterization.

The swelling and GPC studies on the freeze-dried IPN films were carried out according to the same procedures as described in Sections 3.2 and 3.3, except for the replacement of 0.1% sodium azide aqueous solution with a solution of 50% Fetal Bovine Serum (CanSera, Rexdale, ON) and 0.1% sodium azide in deionized water. The swelling was also conducted for some IPN films, prepared by the aging method before they were freeze-dried.

The swelling and GPC studies were carried out as described in Sections 3.2 and 3.3, respectively, except for substituting the 0.1% sodium azide aqueous solution with a solution made of 50% Fetal Bovine Serum (CanSera, Rexdale, ON) and 0.1% sodium azide in deionized water. This solution is referred to as 50% fetal bovine serum. Swelling was also measured for some of the washed IPN films prepared using an aged methacrylated gelatin solution as described in Section 3.4.2.

Mechanical tests were conducted on the freeze-dried films re-hydrated in the 50% fetal bovine serum. The films were cut into strips of 2 cm long × 1 cm wide × 0.2 cm thick. The force and elongation at break point were measured using a Zwick materials testing machine (TC-FR005TN.A50, Zwick USA, Kennesaw, GA, USA) at a deformation rate of 50 mm/min. The tensile failure stress and strain parameters were calculated, respectively, as the force at the break divided by the cross-section area, and as the elongation at the break divided by the initial length of the IPN film.

4.1 Extent of methacrylation

As discussed in Section 3.4.1, gelatin crosslinking took place via the polymerization of methacrylate groups. Therefore, a gelatin with a higher degree of methacrylation would be more crosslinked and subsequently more stable.

Figure 26 shows a higher swelling stability (i.e., maintenance of hydration over a longer period of time) of the IPN film prepared from gelatin with a higher degree of methacrylation. This is consistent with the above stability study in the aqueous solution (Fig. 19), and is due to an increased crosslinking of the gelatin resulting from increased methacrylation.

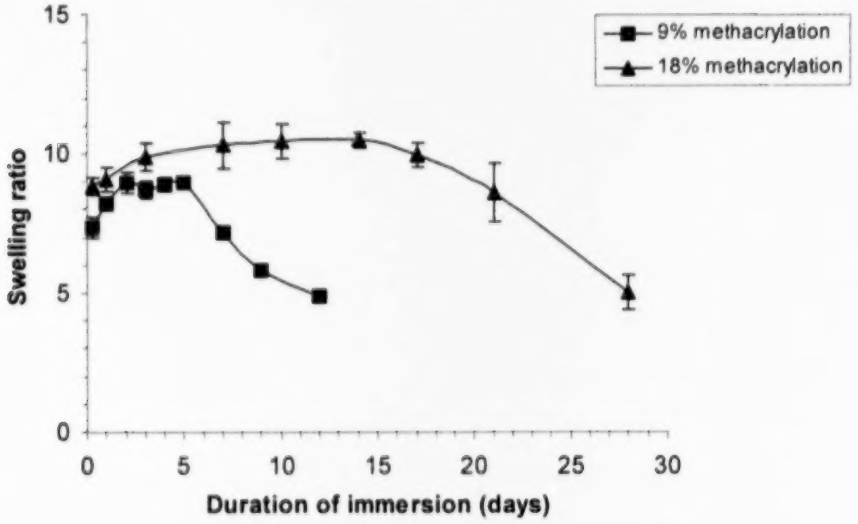

Figure 26. Changes in absorbency of freeze-dried gelatin-HydroThane™ IPN films immersed in 50% fetal bovine serum maintained at 37°C for up to 28 days. Data with error bars represent means ± standard deviation (n=3), otherwise n=1. Films were prepared with methacrylated gelatin with 9 and 18% degree of methacrylation, respectively.

4.2 Concentrations of the photo-initiator

Figure 27 depicts the expected effect of the photo-initiator (Irgacure 651) concentration on the swelling stability. It appears that the IPN films prepared using 5 and 10 wt% Irgacure 651 solutions showed a decline in swelling after a 5-day immersion in the 50% fetal bovine serum at 37°C, while the IPN film prepared at a higher Irgacure concentration had minimal changes in swelling throughout the study. Since the photo crosslinking reaction involved in the preparation of our gelatin-HydroThane™ IPN films was initiated by the UV-decomposition of the photo-initiator Irgacure 651, one could speculate that its relative concentration in the pre-

IPN solution could also affect the IPN formation and its properties: the higher the photo-initiator concentration, the higher the initiation radical yield, which may, in turn, lead to a higher crosslinking and swelling stability. Moreover, crosslinking is needed to prevent the phase separation between the two components and reduce the domain size, leading to improvement in stability and mechanical strength [61]. Although it appears that the IPN film prepared at the lowest Irgacure 651 concentration exhibited a highest swelling ratio for the period of our intended application, its strength might not be high enough for the best performance of the biomaterial as a wound dressing. Therefore, an intermediate concentration of the photo-initiator is recommended.

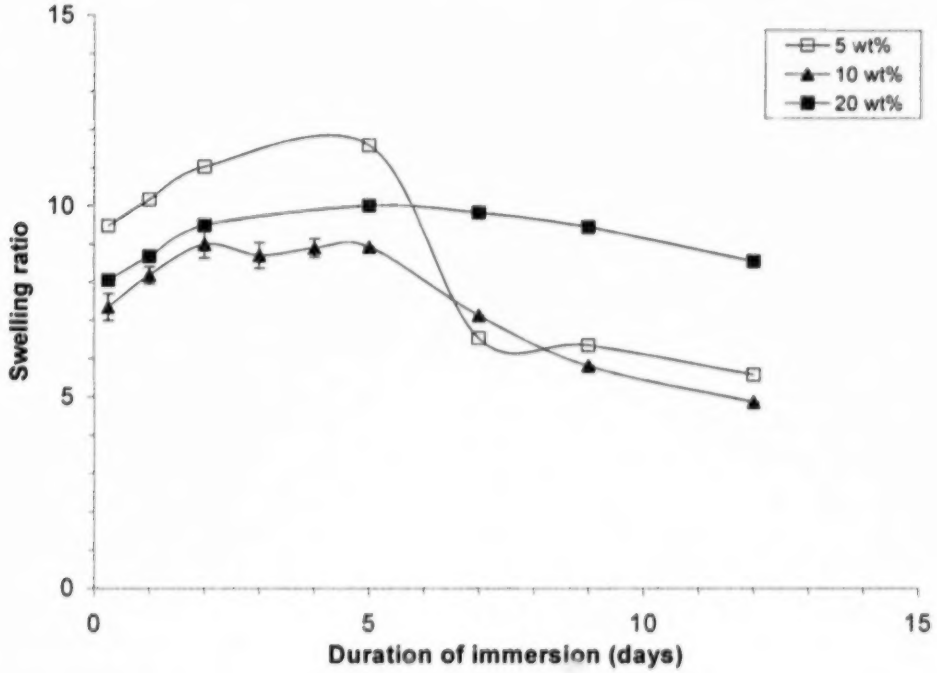

Figure 27. Changes in absorbency of freeze-dried gelatin-HydroThane™ films immersed in 50% fetal bovine serum maintained at 37 ℃ for up to 12 days. Films were prepared with a pre-IPN solution containing various amounts of the photo cross-linker Irgacure 651. Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

4.3 Aging methacrylated gelatin solutions

Figure 28 shows the effect of aging period on the hydration of the IPN films during the washing procedure. Our data show that IPN films prepared using a methacrylated gelatin solution aged for either 2 or 3 weeks had a greater swelling than those prepared using a

solution aged for only one week. The effect was observed at the very beginning with a 40% reduction in hydration on day 5 for the film prepared from a 1-week aged solution. Longer aging times at a solution concentration of 18 wt% were not investigated, since the 4-week aged solution became so viscous that it was difficult to dilute it, therefore resulting in an incomplete mixing with HydroThane™ to form the IPN. The result indicates that although aging may increase physical crosslinking of gelatin through the formation of a helix structure [43], and subsequently reduce the domain size, it may also reduce mixing between the two polymer components. Therefore, 2-3-week aging at 18% concentration is considered the optimum aging time at solution concentration of 18% for the stability of our IPN films.

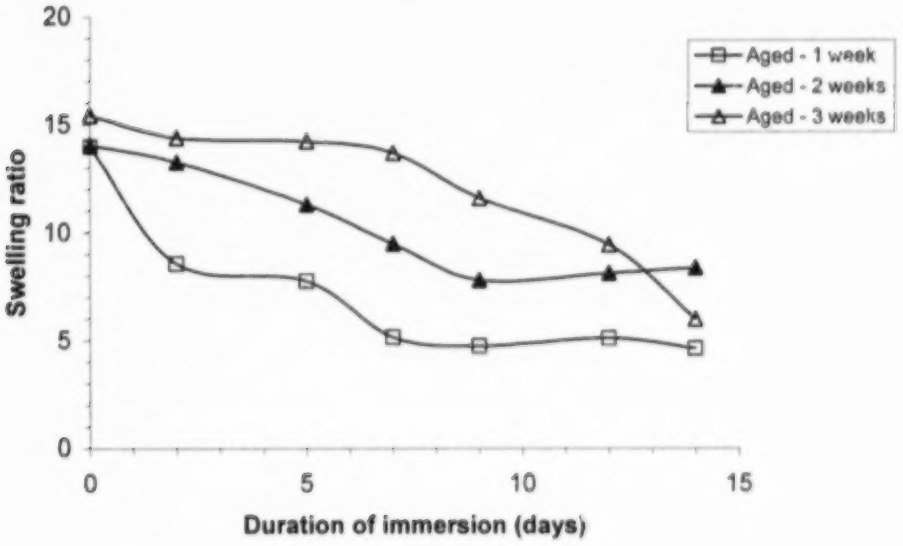

Figure 28. Changes in absorbency of gelatin-HydroThane™ IPN films immersed in 50% fetal bovine serum maintained at 37 ℃ for up to 14 days. Films were prepared with aged methacrylated gelatin solutions, being prepared at 18 wt%, stored for up to three weeks at room temperature, and diluted to 7.5 wt% prior to use. Data represent n=1 per time interval.

Figure 29 shows the effect of aging the methacrylated gelatin solutions on the swelling stability of the IPN films. We compared the hydration of the IPN films prepared using a fresh methacrylated gelatin solution with that of an IPN film prepared using the aging method, where the methacrylated gelatin solution was aged at 18% for 3 weeks and then diluted to 7.5% to prepare the IPN film. Our results show that the non-aged films swelled within the first 2 days, maintaining a swelling ratio of approximately 9 over the ensuing 3 days, before showing a reduction in swelling. In contrast, the aged film swelled continuously for 8 days before a reduction in swelling was observed. Furthermore, the rate of decline in swelling observed for the non-aged film was higher than that of the aged film, the overall reduction

being twice greater for the non-aged than the aged film. These findings are consistent with our previous observation of maintenance of the hydration level when films prepared using the aged solution (Fig. 21) were immersed in an aqueous solution. Moreover, it is likely that the smaller domain size in the aged IPNs (Fig. 22) played a role in these hydration patterns.

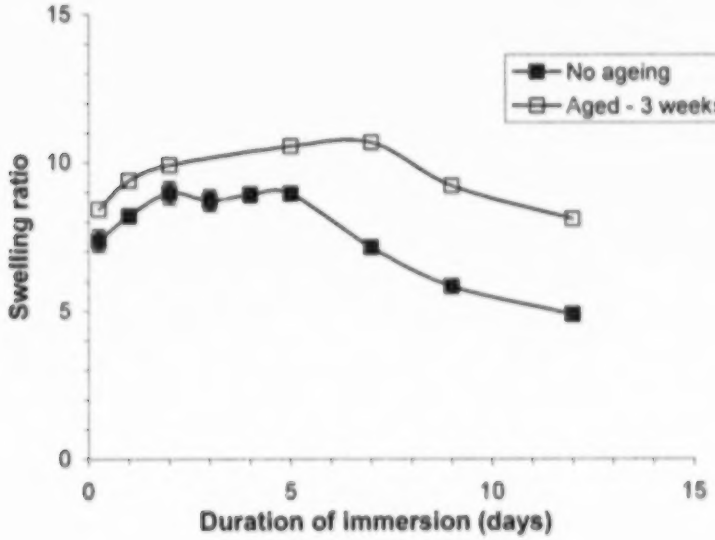

Figure 29. Changes in absorbency of freeze-dried gelatin-HydroThane™ IPN films immersed in 50% fetal bovine serum maintained at 37 ℃ for up to 12 days. Films were prepared with either a fresh (non-aged) or an aged diluted methacrylated gelatin solution, the latter solution being prepared at 18 wt%, being stored for up to three weeks at room temperature, and being diluted to the same concentration as the fresh solution (7.5 wt%) prior to use. Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

4.4 Freezing of the pre-IPN solutions

As described in Section 3.4.3, the freezing method of IPN preparation included freezing the mixed polymer solutions at approximately 0, -70, and -200°C prior to initiating the photo crosslinking process, to reduce the phase separation between gelatin and HydroThane™ during the IPN formation. Figure 30 shows that all IPN films prepared using this method maintained their hydration levels for a longer period of time than the IPN film prepared without any freezing. This greater stability is consistent with the swelling studies in the aqueous solution (Fig. 23), possibly due to the smaller domains in the IPN films prepared using the freezing method as observed by light microscope (Fig. 24).

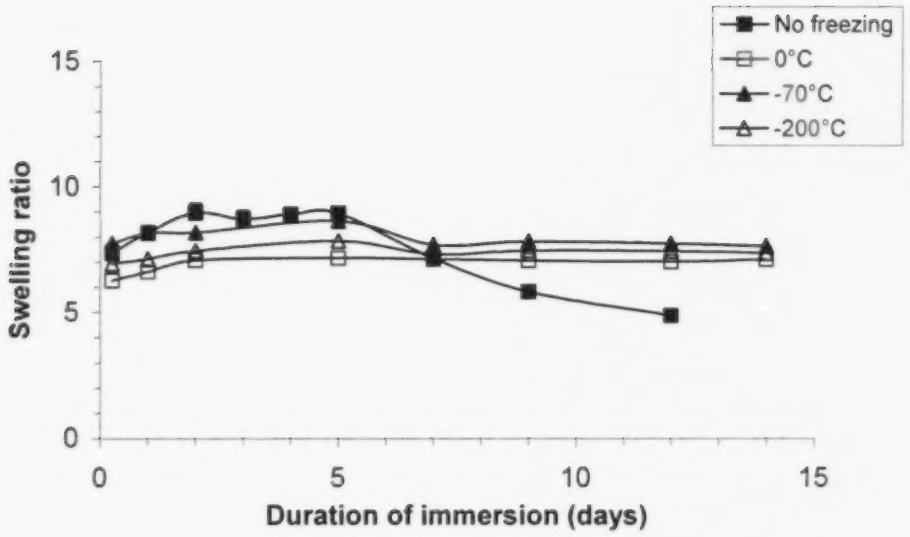

Figure 30. Changes in absorbency of freeze-dried gelatin-HydroThane™ IPN films immersed in 50% fetal bovine serum maintained at 37°C for up to 14 days. All films were prepared from pre-IPN solutions that were kept at various temperatures for at least 15 min prior to initiating the photo cross-linking process. Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

Figure 31 shows that the temperature at which the mixed gelatin and HydroThane™ solution was frozen had an effect on the swelling stability of the IPN films. Although all films maintained their hydration for about 5 days, the magnitude of the reduction in hydration observed after a 15-day immersion varied, averaging approximately 6%, 50% and 60% at 0, -200 and -70°C, respectively. While freezing the pre-IPN mixture reduced the domain size (Fig. 24) and subsequently resulted in an increased stability, the low temperatures also decreased the rate at which the crosslinking reaction proceeded, leading to the decrease in the stability. The results would suggest that the optimal stability of our IPN biomaterial is achieved at 0°C.

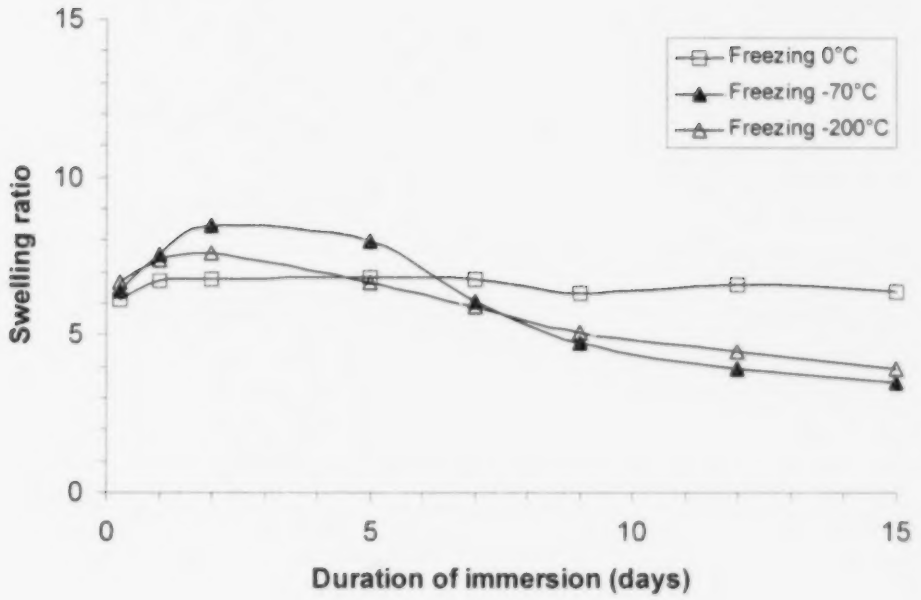

Figure 31. Changes in absorbency of freeze-dried gelatin-HydroThane™ IPN films immersed in 50% fetal bovine serum maintained at 50 °C for up to 15 days. Films were prepared from pre-IPN solutions that were kept at various temperatures for at least 15 min prior to initiating the photo cross-linking process. Data represent n=1 per time interval.

4.5 Effect of serum temperature

The temperature at which the IPN films were incubated had a marked effect on the swelling stability (Fig. 32). Although the IPN films exhibited comparable swelling for up to 4 days, irrespective of the temperature of the 50% fetal bovine serum, there was a tendency towards a temperature-dependent reduction in the absorbency of the biomaterial after a 4-day immersion. At room temperature, the IPN films could maintain the hydration level over the 12-day investigation period. In contrast, they tended to decrease in swelling after 6 days of immersion at 37°C and 4 days of immersion at 50°C, respectively. The decrease in swelling stability with increasing temperature is due to increased gelatin hydrolysis and solubility. This is consistent with our GPC results, showing a decrease in stability with the medium temperature and with reported effects of temperature on the swelling stability of a biomaterial [53].

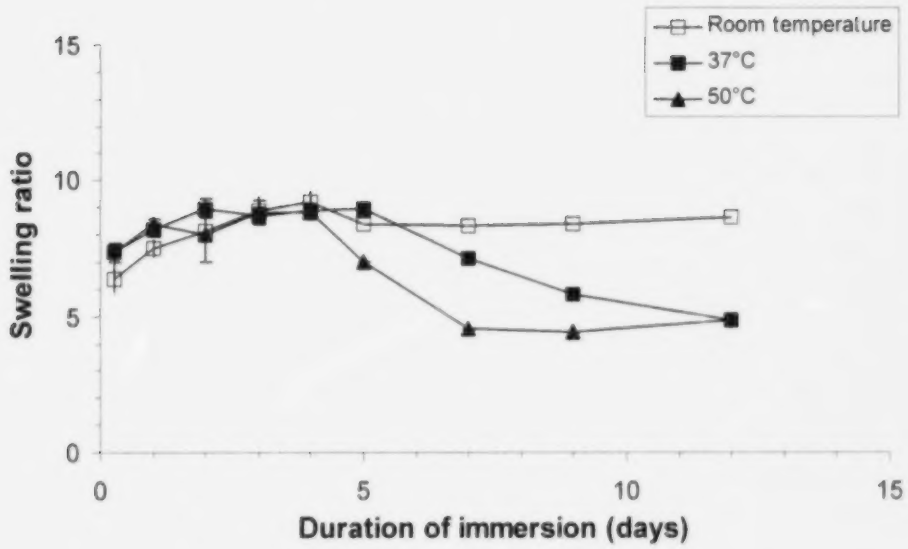

Figure 32. Changes in absorbency of freeze-dried gelatin-HydroThane™ films immersed in 50% fetal calf serum maintained at various temperatures for up to 12 days. Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

4.6 HydroThane™ stability

HydroThane™ is a brand name for a family of thermoplastic polyurethanes typically composed of hard and soft segments. The biostability of polyurethane biomaterials has been reviewed [62]. It appears that the hydrolytic degradation of polyurethanes involves the cleavage of ester or ether bonds in the soft segment, and urethane bonds in the hard segment. The extent of the degradation of HydroThane™ will also depend on its chemical and physical structures [63, 64]. No detectable residuals were detected using our GPC method when one HydroThane™ film was immersed in the aqueous solution for up to 34 days. This finding is consistent with that of a minimal change in the dry weight of the film after the 34-d immersion (data not shown).

As the HydroThane ™ component confers strength and elasticity to the IPN film, the impact of its stability on the mechanical properties of the film was investigated. Figures 33 and 34 show no statistically significant changes in the tensile strength and strain capability of the film with increasing either the duration of the immersion or the temperature of the medium, although a decrease in swelling of the IPN prepared using the same methacrylated gelatin (9% methacrylation) was observed at 37°C, as shown in Figure 26. This higher mechanical stability compared to the swelling stability may be because the stress and strain were mainly

provided through the more stable HydroThane™ component, while swelling resulted mainly from the gelatin component, which degraded and dissolved out.

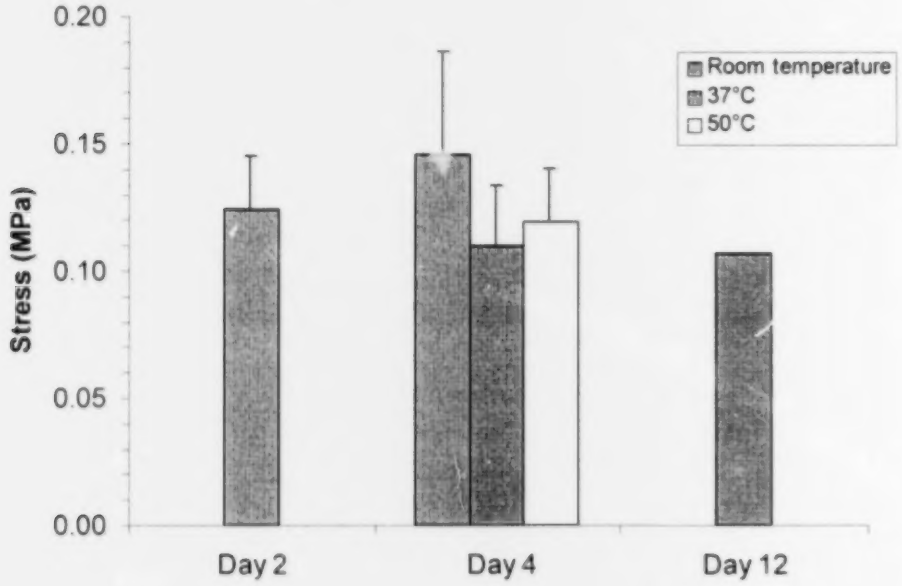

Figure 33. Stress of freeze-dried gelatin-HydroThane™ IPN films immersed in 50% fetal bovine serum maintained at different temperatures. Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

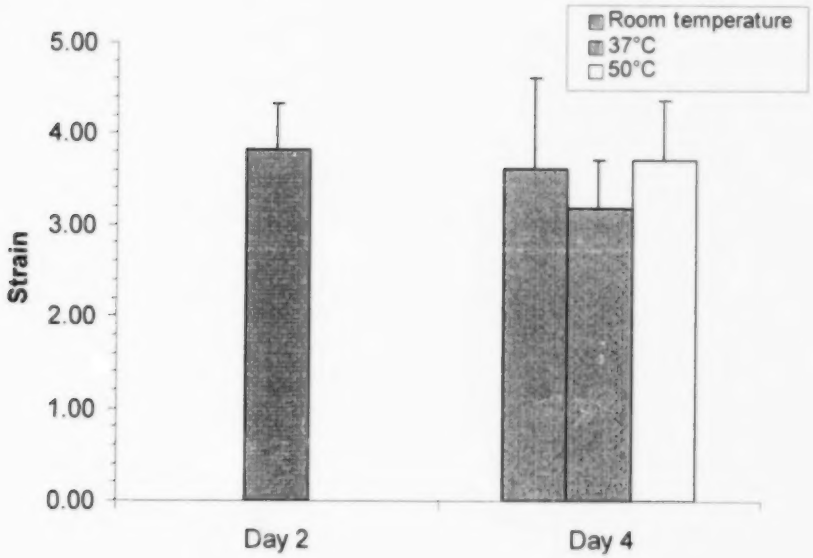

Figure 34. Strain of freeze-dried gelatin-HydroThane™ IPN films immersed in 50% fetal bovine serum maintained at different temperatures. Data are expressed as means ± standard deviation (n=3).

5. Summary of key findings

In this study, we first developed a GPC method to measure the molecular weight and composition of gelatin, and also characterize the stability of native gelatin in aqueous solutions. Our data show that the molecular weight of gelatin is heterogeneous. The high-molecular-weight fraction of gelatin (i.e., γ chains) seems to be most susceptible to hydrolysis perhaps through breakdown of the intramolecular crosslinkage.

Our initial studies enabled us to control the quality of the methacrylation of gelatin and further investigate the stability of the crosslinked gelatin and gelatin-HydroThane™ IPN films. For example, the temperature-dependent hydrolysis of gelatin appears to be related to the stability of the IPN biomaterials in various media at different temperatures. This was further confirmed by comparing the degradation of native gelatin with the gelatin loss from the biomaterials (Fig. 35). The percentage of native gelatin hydrolyzed through breakdown of chemical links in the γ chains was calculated using the following equation: $(M_0-M_t)/M_0$, where M_0 and M_t represent the amounts of the γ-chain fraction at time 0 and time t, calculated from the area of peak 1 under the chromatogram (Fig. 9). It was used to estimate the gelatin degradation as the hydrolysis mainly took place through the breakdown of the γ chains. The gelatin loss from the crosslinked gelatin and IPN films was calculated as a percentage of cumulative amount of gelatin residuals in the solution over initial amount of gelatin in the film as described in Section 3.2. The gelatin loss was about 46% and 50% after immersion of the crosslined gelatin and IPN films for 31 and 34 days at 37°C, respectively (Fig. 16).

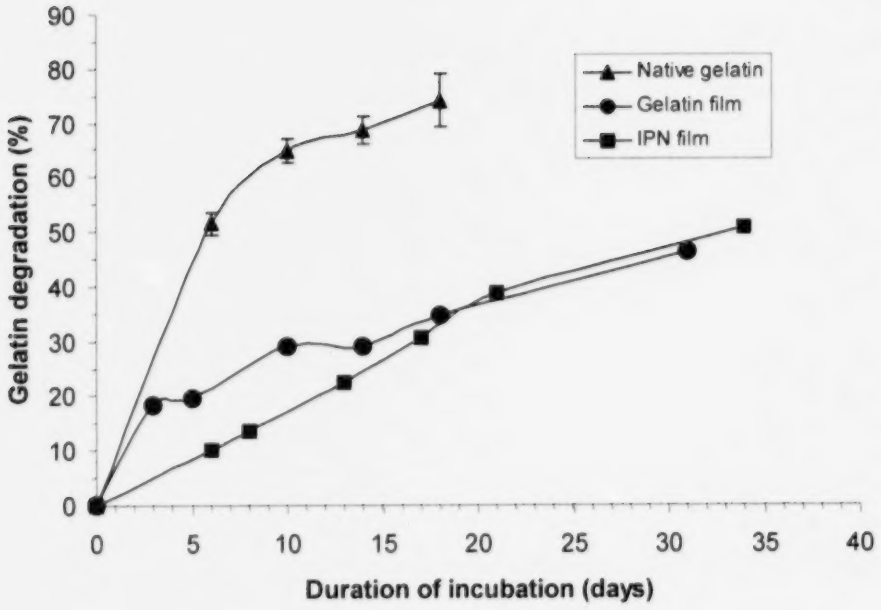

Figure 35. Stability of native gelatin, freeze-dried photo-crosslinked gelatin and gelatin-HydroThane™ IPN films in a 0.1% sodium azide aqueous solution maintained at 37 ℃ for up to 34 days. Data with error bars represent means ± standard deviation (n=3), otherwise n=2.

Figure 35 shows that the rate of hydrolysis of native gelatin is greater than that of the crosslinked gelatin in either the IPN or gelatin film. These data are consistent with the report demonstrating chemical and physical crosslinking reduced gelatin degradation [27]. In addition, similar degradation profiles were observed among native and crosslinked gelatin, implying correlation in stability between native gelatin and IPN gelatin.

Although gelatin loss was observed from the freeze-dried IPN biomaterials, their swelling stability was maintained for at least 4 days, a time interval corresponding to the maximum period of application of our biomaterial as a wound dressing. The IPN films exhibited higher stability and hydration in the aqueous solution than in the serum (Fig. 36), likely due to differences in the composition and pH of the solutions [59, 60].

Figure 37 indicates that absorbency of IPN films was reduced after the freeze-drying process. This may be due to the fact that freeze-drying caused a collapse of the gelatin network and formation of strong intermolecular hydrogen bonds within the network [65].

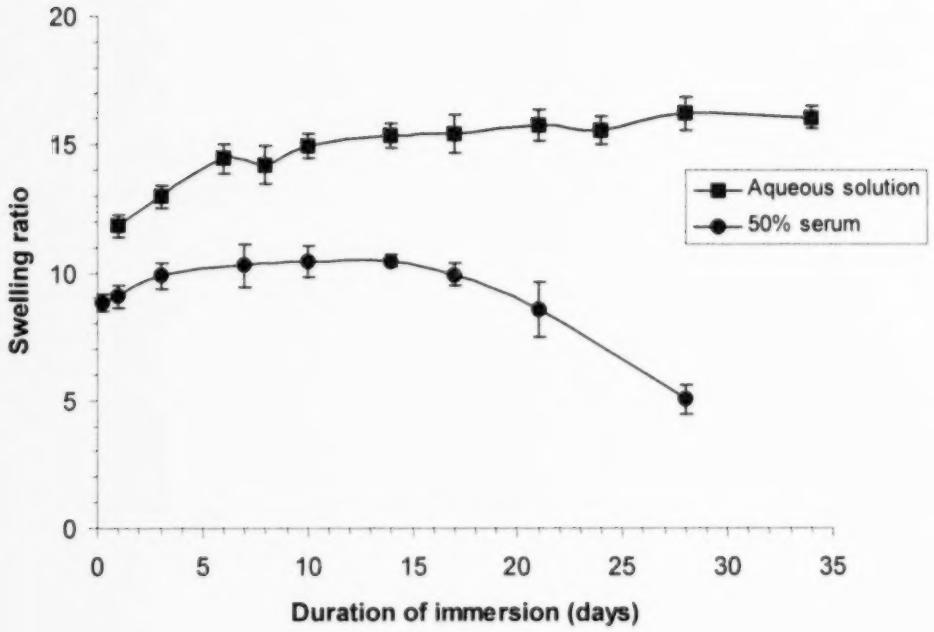

Figure 36. Re-hydration characteristics of freeze-dried gelatin-HydroThane™ IPN films immersed in a 0.1% sodium azide aqueous solution or 50% fetal bovine serum maintained at 37 ℃ for up to 34 days. Films were prepared using 18% methacrylated gelatin. Data are expressed as means ± standard deviation (n=3).

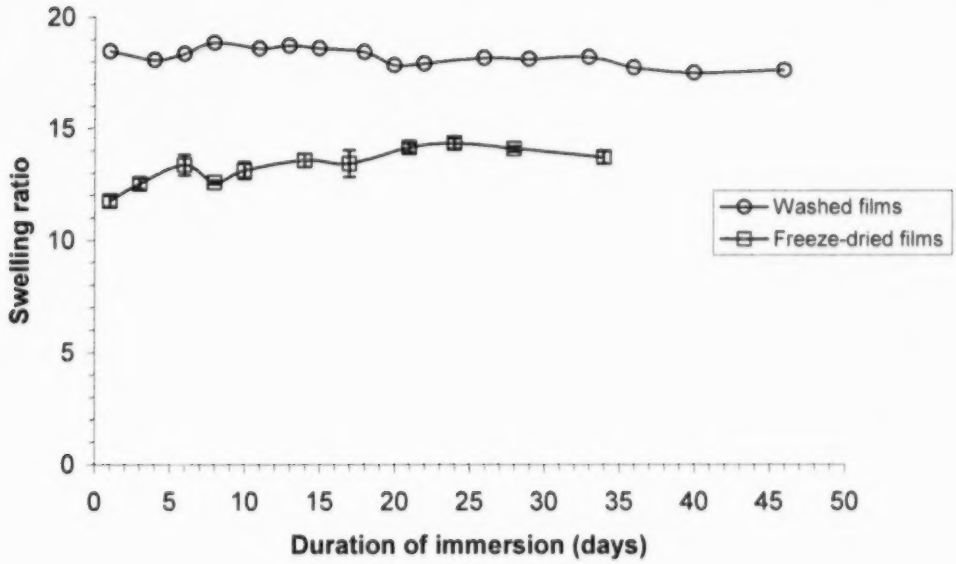

Figure 37. Swelling characteristics of gelatin-HydroThane™ IPN films immersed in a 0.1% sodium azide aqueous solution maintained at room temperature for up to 46 days. Swelling ratio was measured before (washed films) or after (freeze-dried films) the freeze-drying process. Films were prepared using 18% methacrylated gelatin. Data with error bars represent means ± standard deviation (n=3), otherwise n=1.

The focus of our work was to address the stability and hydration issues surrounding the development of the gelatin-HydroThane™ IPN biomaterials for use as component of a wound dressing for front-line casualty care. The correlation between the preparation conditions of the biomaterials and their *in vitro* stability and hydration in various media is summarized in Table 2. Although different stability and hydration profiles were observed between gelatin-HydroThane™ films immersed in a 0.1% sodium azide solution and in a 50% fetal bovine serum (Fig. 36) as well as between the washed and freeze-dried IPN films (Fig. 37), they were all affected by the preparation parameters in a similar manner regardless of the type of media and wash/freeze-drying processes.

Table 2. Effects of preparation parameters on the stability and hydration of gelatin-HydroThane™ IPN films in aqueous and serum solutions.

Type of IPN films	Parameter	Stability		Hydration		Recommendation
		0.1% sodium azide solution	50% fetal bovine serum	0.1% sodium azide solution	50% fetal bovine serum	
Washed	Extent of methacrylation	++++	N/A	×	N/A	Increase
	Use of crosslinker	+++	N/A	---	N/A	Not required
	Aging	+++	++	-	-	Not required
	Freezing	++	N/A	--	N/A	Not required
Freeze-dried	Extent of methacrylation	N/A	++++	N/A	≅	Increase
	Photo-initiator concentration	N/A	+	N/A	≅	Intermediate concentration
	Aging	N/A	++	N/A	≅	Not required
	Freezing	N/A	++	N/A	-	Not required

N/A Not Available. The symbols +, - and ≅ denote positive, negative and minimal effects for each parameter. The number of symbols for a given parameter arbitrarily indicates its overall impact.

In summary, all freeze-dried IPN films re-hydrated well and maintained a swelling ratio of at least 9 for approximately 3 days, even when the medium was maintained at 50°C. Increasing the extent of methacrylation of gelatin and photo-initiator concentration led to more stable films due to an increased crosslinking of gelatin. The IPN films prepared using the aging and freezing methods were more stable due to their smaller IPN domains. The extent of methacrylation exerted the greatest impact on stability, followed by the presence of crosslinker, aging of the methacrylated gelatin, freezing of the pre-IPN mixture, and finally the concentration of photo-initiator in the pre-IPN mixture. It is noteworthy that due to the most negative effect of addition of the crosslinker on the swelling as observed in the aqueous solution, no further study of its effect was conducted in the serum.

Considering that the IPN films were mostly stable during an application period no more than 4 days, we used hydration as a criterion to standardize the preparation conditions. The use of 18% methacrylated gelatin is recommended as a higher degree of methacrylation improved the stability without compromising the absorbency of the biomaterials. All the other factors tested (i.e., presence of crosslinker, concentration of photo-initiator, aging the methacrylated gelatin solutions and freezing the pre-IPN solutions) may either reduce the absorbency or add additional steps to the preparation of our IPN films without any significant benefits to the absorbency. It is noteworthy that other requirements such as mechanical properties must be considered for optimizing the composite biomaterial.

6. Conclusions

The present report demonstrates that the gelatin component of our biopolymer-elastomer IPN biomaterial undergoes temperature-dependent degradation upon its immersion in both aqueous and serum solutions. However, the total gelatin loss was relatively small over the period of application for our intended use for up to 4 days, and did not reduce the overall absorbency of the IPN biomaterial. Furthermore, the biomaterial could still maintain its swelling stability for more than 30 days in the aqueous solution. While the biomaterial showed lower swelling stability in the serum-containing medium than in the aqueous solution, it could still maintain its swelling level in the serum for at least 4 days even at 50°C, and the swelling stability was further increased by increasing the degree of gelatin methacrylation and photo-initiator concentration. The stability in both aqueous and serum solutions was decreased with increasing incubation temperature. In addition, there was neither a measurable degradation of the elastomer nor an impact on the mechanical properties of the films.

To achieve the prevention of a phase-separation of the gelatin and HydroThane™ components in our IPN, We developed two novel methods for preventing namely, aging (by storage) of the methacrylated gelatin solution and freezing of the pre-IPN solution prior to the preparation of the IPN films. Although our results revealed that the two methods reduce phase separation, thereby improving the biomaterial's swelling stability, neither of these methods proved useful to significantly increase the hydration of our IPN biomaterial. In this respect, increasing the extent of gelatin methacrylation appeared the simplest method to increase the stability while maintaining the absorbency of the IPN biomaterial to levels comparable to those of commercially available wound dressings.

While the present series of studies contributed to further our understanding of the factors affecting the physicochemical properties of our biomaterial, a number of issues remain to be addressed. For example, the relationship at the molecular level: the composite structures (e.g., helix structures and hydrogen bonding); the effect of the preparation conditions on the morphology; and, the physicochemical properties of the IPN biomaterial should be investigated. Indeed, the cross-linking process during formation of our IPN might be a competing process. On the one hand, cross-linking is required to both prevent the phase-separation between gelatin and HydroThane™ and reduce the domain size, thus leading to the biomaterial's improved stability, mechanical properties, and drug release. On the other hand, excessive cross-linking may promote phase-separation as a result of decreased polymer chain mobility and mixing, and increase the domain size.

We envisage configuring the gelatin-HydroThane™ IPN biomaterial into a tri-dimensional mesh or puff that will be applied on deep, hemorrhagic wounds. Studies must be carried out to determine the best strategy for incorporating a hemostatic agent in the gelatin-HydroThane™ IPN mesh and puff without compromising its absorbency and mechanical properties. Furthermore, the formulation of the biomaterial may need to be further optimized if the procedures used for the preparation of the IPN mesh and puff adversely affect the physicochemical properties of the biomaterial. Nevertheless, we believe that the current formulation of the gelatin-HydroThane™ IPN films has been optimized, allowing the *in vitro*

and *in vivo* bactericidal properties of the biomaterial to be assessed. In summary, our studies have provided new approaches for the preparation and characterization of IPN biomaterials.

7. References

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List of symbols/abbreviations/acronyms/initialisms

DMAP	Dimethylaminopyridine
DMSO	Dimethyl sulfoxide
DRDC	Defence Research & Development Canada
GPC	Gel Permeation Chromatography
$HiMw_{gelatin}$	High-molecular-weight-fraction of gelatin
h	Hour
IPN	Interpenetrating Polymer Network
$LoMw_{gelatin}$	Low-molecular-weight fraction of gelatin
Min	Minute
Mg	Milligram
MAAH	Methacrylic Anhydride
Mw	Molecular weight
N/A	Not available
Nm	Nanometer
PBS	Phosphate Buffered Saline
RT	Room Temperature
SD	Standard Deviation
TEA	Triethylamine
UV	Ultraviolet

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(U) This report describes the stability and hydration properties of gelatin and gelatin−HydroThaneTM composite biomaterials in different media. A Gel permeation chromatography (GPC) method and swelling measurements were applied to examine the stability of the tested biomaterials. The degradation of gelatin in distilled water and 0.1% sodium azide aqueous solution was confirmed by the GPC study. The degradation in terms of changes in both molecular weight and its distribution was increased with increasing temperature. Samples of gelatin−HydroThaneTM composite biomaterials were prepared by photochemical crosslinking of gelatin and HydroThaneTM in dimethylsulfoxide solution under different conditions. Results of the stability and hydration studies in the aqueous solution demonstrated that the freeze−dried composite biomaterial exhibited relatively stable swelling for more than 30 days, but a loss of the gelatin component occurred, approximately by 10% at room temperature and 50% at 37„aC. Similar swelling study in a serum−containing medium indicated that the composite biomaterial would maintain the hydration stability for its potential application in a wound dressing. A number of key factors in the preparation of the biomaterial were investigated to optimize its hydration properties. The composite biomaterial showed good mechanical properties, primarily conferred by the more stable HydroThaneTM component. The results of this study provide a better understanding of the physicochemical properties of a gelatin−HydroThaneTM composite biomaterial, which holds much promise for its further development into a dressing for wound management and other biomedical applications.

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(U) Hydrogel; Elastomer; Interpenetrating Polymer Network; Wound dressing

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